SEX DIFFERENCES IN THE EFFECTS OF TWO-HIT STRESS ON THE STRUCTURE AND FUNCTION OF RAT MEDIAL PREFRONTAL CORTEX.

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Kelly M. Moench

SEX DIFFERENCES IN THE EFFECTS OF TWO-HIT STRESS ON THE STRUCTURE AND FUNCTION OF RAT MEDIAL PREFRONTAL CORTEX.

Stress increases the risk for a number of psychological disorders, including mood and anxiety disorders. Prefrontal cortex dysfunction likely contributes to the cognitive symptoms associated with these disorders. Notably, women are at an increased risk for many stress-related disorders, although the neurobiological mechanisms underlying this enhanced vulnerability have yet to be elucidated. Evidence from rodent models suggests that male rats are susceptible to the effects of chronic stress on the structure and function of medial prefrontal cortex (mPFC), with numerous neurobiological and behavioral changes observed shortly after the cessation of stress. In contrast, female rats often do not exhibit these same deleterious effects of chronic stress. In this dissertation I investigated if chronic stress-induced changes in the structure and function of mPFC persist following the cessation of stress, and if sex differences in the initial response to chronic stress result in sex-specific changes following a novel stress challenge. In Experiment 1, I showed that dendritic remodeling of neurons in mPFC is sex-specific during the post-stress period. To assess whether these changes might give rise to functional changes, I developed a "two-hit stress" paradigm in which rats were exposed to chronic stress, given a no-stress rest period, and then exposed to a novel stress challenge (i.e., a "second hit"). Using this paradigm, I demonstrated in Experiment 2 that males, but not females, have a persistent reduction in novel stress-induced neuronal activation in mPFC. I then showed in Experiment 3 that, behaviorally, male rats have a deficit in extradimensional set-shifting immediately after chronic stress. This deficit is ameliorated following a rest period, and does not re-emerge following the second "hit." In contrast, female rats only show a behavioral deficit following the second "hit." Finally, in Experiment 4 I began to investigate mechanisms that may contribute to these sex differences and showed that there are sex-specific changes in the expression of genes related to glutamatergic

and GABAergic neurotransmission in mPFC that occur during the post-stress period. These data suggest that chronic stress likely leads to the recruitment of sex-specific stress adaptation mechanisms that contribute to sex differences in response to subsequent stress exposure.

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Chapter 1:

Introduction

Stress, Sex, and Dysfunction of Prefrontal Cortex in Major Depressive Disorder

Stress is a major risk factor for a number of psychopathologies, including anxiety and mood disorders (Risch et al., 2009; Lian et al., 2014). The prevalence of many of these disorders is markedly higher in women than men (Solomon and Herman, 2009; Cover et al., 2014). Additionally, there are sex differences in the severity, persistence and symptomatology of many of these disorders. These differences are best-documented in major depressive disorder (hereafter referred to as "depression"). For example, women are more likely to experience recurrent depression (Kessler et al., 1994; Kornstein et al., 2000), have atypical depressive symptoms (e.g., hypersomnia, weight gain; Angst et al., 2002), and have comorbid anxiety disorders (Breslau et al., 1995). In contrast, men with depression are more likely to have comorbid substance use disorders (Najt et al., 2011). Additionally, antidepressant efficacy can differ between men and women such that men typically respond better to tricyclic medications, whereas women may benefit more from selective serotonin reuptake inhibitors (reviewed in LeGates et al., 2019). Despite the vast amount of empirical and epidemiological evidence demonstrating sex differences in the prevalence and presentation of stress-linked disorders, the neurobiological mechanisms underlying sex-specific vulnerabilities are only beginning to be elucidated.

Structural and functional abnormalities of prefrontal cortex (PFC) – a region critical for executive functioning and emotion regulation – are well-documented in individuals with depression. For example, depression is associated with decreased gray matter volume (Du et al., 2012; Sacher et al., 2012), which is more prominent in chronic depression sufferers compared to those in sustained remission (Salvadore et al., 2011). Further, decreased cortical volume in the PFC is inversely correlated with feelings of sadness, fatigue, and worry (Lener et al., 2016). Postmortem studies of individuals with depression show a decrease in pyramidal neuron soma

size (Rajkowska et al., 1999), dendritic spine density reduction on pyramidal neurons (Kang et al., 2012), a reduction in glial cell density (Rajkowska et al., 1999), and alterations in inhibitory neuron density within dorsolateral and medial PFC (Rajkowska et al., 2007).

Functional changes in PFC are also present in depression. For instance, PFC baseline activity is often blunted in individuals with depression (Drevets et al., 2008). This reduction in PFC activity may contribute to reduced inhibitory control over the amygdala, resulting in a diminished ability to process emotional stimuli (Gotlib et al., 2004; LaBar and Cabeza, 2006; Joormann and Gotlib, 2010). Additionally, it is estimated that as many as two-thirds of individuals with depression suffer from cognitive deficits that arise from dysfunction of PFC (Butters et al., 2004; Afridi et al., 2011). These can include impairments in working memory, cognitive flexibility, and attention (Bora et al., 2012; Rock et al., 2014). Further, cognitive symptoms can persist even when other symptoms (e.g., depressed mood) have abated (Jaeger et al., 2006; Hasselbalch et al., 2011), which likely impacts the long-term psychosocial functioning of these individuals. Thus, elucidating the mechanisms underlying prefrontal dysfunction in stress-sensitive disorders could contribute to novel treatment options not only for individuals with current diagnoses, but also for individuals in which mood symptoms have remitted, but cognitive symptoms persist.

Although PFC-related changes in depression are well-documented, few studies have examined if these changes are sex-specific. This is a critical area of research given the disparity in prevalence, severity of symptoms, and treatment outcomes between men and women with depression. Recent studies have begun to elucidate possible neurobiological mechanisms by which these sex differences in depression may arise. These putative mechanisms include aberrant glutamatergic neurotransmission in PFC, as postmortem analyses have indicated women, but not men, with depression had higher expression of NMDA and AMPA receptor-related genes compared to healthy controls (Gray et al., 2015; Labonté et al., 2017). Others have shown that GABAergic neurotransmission may also differ between men and women in depression, as somatostatin and related genes are downregulated to a greater extent in postmortem tissue of

women (Seney et al., 2013). Serotonergic signaling may also be altered in a sex-specific manner in depression, as the expression of the serotonin receptor $5HT_{1A}$ is reduced in PFC of women, but not men, with depression (Szewczyk et al., 2009). This may also partly explain the greater efficacy of SSRI treatment in women. Although these postmortem studies cannot determine whether changes in PFC precede depression or are part of the resulting pathology, they do point to potential mechanisms of PFC dysfunction and possible explanations for differences in symptom profiles between men and women.

The Effects of Stress on the Healthy Adult Human Brain

Stress is only one of many risk factors for the development of psychiatric disorders. Given that a fraction of individuals who experience prolonged and/or severe stressful life events go on to develop stress-related psychological disorders, understanding how the sequelae of post-stress adaptations that occur in the healthy adult brain interact with other known risk factors (e.g., genetic, environmental, lifestyle, etc.) is critical to our understanding of the etiology of these disorders. Few studies have taken this approach, but those that have suggest that there are a number of changes that occur in the structure and function of stress-sensitive brain regions in individuals who experience stress but do not go on to develop stress-linked disorders.

Structurally, prolonged and cumulative stressful life events are associated with decreased gray matter volume in a number of stress-sensitive brain regions. For example, healthy individuals reporting prolonged occupational stress, defined as working 60 to 70 hours per week for several years, had reduced gray matter volume in anterior cingulate cortex and dorsolateral PFC (Blix et al., 2013). Additionally, a greater number of perceived stressful life events is associated with reduced gray matter volume in PFC, insular cortex, anterior cingulate cortex, orbitofrontal cortex, and hippocampus (Gianaros et al., 2007; Papagni et al., 2011; Ansell et al., 2012).

These structural differences likely give rise to functional changes in individuals who have experienced prolonged or severe stress. For instance, enhanced amygdala activation in response

to fearful faces has been observed in veterans following combat exposure (van Wingen et al., 2011). Enhanced amygdala reactivity could result from deficient modulation of the amygdala by PFC, as prolonged occupational stress is associated with reduced functional connectivity between PFC and the amygdala, as well as deficits in attentional control and working memory (Jovanovic et al., 2011). Disrupted frontoparietal connectivity and associated deficits in attentional control have also been found in medical students following one month of academic stress (Liston et al., 2009a). In this case, these changes were absent following a one-month low-stress period, suggesting stress-induced changes in brain structure and function may not be permanent. This likely depends on the length and/or severity of stressful life events. For instance, three years following the 9/11 terrorist attack, enhanced amygdala reactivity was reported in individuals who were in close proximity to the World Trade Center when the attack took place (Ganzel et al., 2007).

These studies suggest that there is a pattern of changes that occur in brain structure and function in non-patient populations following prolonged or severe stressful life events. What is unknown is how this pattern of changes can go awry and result in psychopathology. It is possible that these changes fall on a spectrum and thus simply occur to a greater magnitude in individuals with stress-related disorders. It is also possible that these documented post-stress changes represent normal stress adaption, and that other risk factors may interact with and/or disrupt the course of and/or exacerbate these changes, resulting in pathological outcomes. Although this question is difficult to address within the realm of human research, rodent models of stress can shed light on what short- and long-term changes might be expected to occur in stress-sensitive brain regions and how these change may interact with other known risk factors.

Sex Differences in the Effects of Stress on Medial Prefrontal Cortex Rodents

Stress Effects in Males

Studies in rodents have also demonstrated that stress impacts the structure and function of a number of corticolimbic brain regions, including medial prefrontal cortex (mPFC). In male rats chronic daily restraint stress decreases apical dendritic length and branching of pyramidal neurons in mPFC (Cook and Wellman, 2004; Radley et al., 2004; Radley et al., 2005; Garrett and Wellman, 2009), reduces the density of dendritic spines (Radley et al., 2006; Liu and Aghajanian, 2008; Radley et al., 2008; Hains et al., 2009), and results in a shift in spine maturity from larger, more mature spines to smaller, less mature spines (Radley et al., 2008). Spine maturity is thought to be correlated with plasticity and memory formation such that smaller spines are considered more labile and potentially more critical during the learning process, whereas larger spines are more stable and may be indicative of memory formation (Rochefort and Konnerth, 2012). A reduction in total spine density concurrent with reduced spine size suggests that chronic stress impairs both of these processes. Chronic stress also reduces *ex vivo* stimulus-evoked excitatory postsynaptic potentials (Liu and Aghajanian, 2008) as well as the surface expression of glutamatergic receptor subunits (Wei et al., 2014). On the other hand, the expression of GABAergic markers of neurotransmission appears to be unaffected (Shepard et al., 2016; Shepard and Coutellier, 2018). Together, these changes are likely responsible for reduced glutamate transmission (Jett et al., 2017) and impaired long-term potentiation (LTP) induction in mPFC (Cerqueira et al., 2007; Goldwater et al., 2009; Zheng and Zhang, 2015). LTP is thought to be a crucial mechanism by which learning and memory occurs (Nicoll, 2017). This, combined with alterations in spine lability, suggests that chronic stress alters mechanisms of mPFC-dependent learning, resulting in impaired performance on tasks mediated by mPFC.

Indeed, following a variety of chronic stress paradigms, males have impaired performance on tasks that are mPFC-dependent. For instance, following 21 days of restraint stress, male rats have poorer working memory performance as measured by a temporal order task (Wei et al.,

2014) and delayed alternation (Hains et al., 2009). This same stress paradigm also results in deficits in extradimensional set-shifting (Liston et al., 2006), another behavioral task dependent on mPFC (Birrell and Brown, 2000). Others have demonstrated that just 7 days of repeated restraint (Nikiforuk and Popik, 2011, 2013) and 14 days of chronic unpredictable stress also disrupt extradimensional set-shifting in male rats (Bondi et al., 2008; Bondi et al., 2010; Jett et al., 2017). Altogether, these studies suggest that mPFC of male rodents undergoes substantial changes in both structure and function as a result of chronic stress exposure.

Stress Effects in Females

A small but growing number of studies suggest there are notable sex differences in the effects of stress on structure and function of mPFC. Unlike in male rats, repeated restraint stress induces dendritic outgrowth in females (Garrett and Wellman, 2009). Further, the expression of glutamatergic receptor subunits is also unaffected by chronic stress (Wei et al., 2014). Instead, unlike in males, the GABAergic system appears to undergo more substantive changes as a result of chronic stress (Shepard et al., 2016; Shepard and Coutellier, 2018). There are also differences in the pattern of DNA methylation in mPFC of male and female rats following 14 days of twice-daily elevated platform stress (Mychasiuk et al., 2016), although the functional significance of this difference is unclear.

Unlike the pattern of general disruption in mPFC-dependent tasks in males, these same tasks are largely unaffected by chronic stress in females. For instance, 21 days of repeated restraint stress does not disrupt temporal order working memory (Wei et al., 2014). Similarly, extradimensional set-shifting is intact following sub-chronic social defeat stress (Snyder et al., 2015). This effect may differ by species, as 14 days of chronic unpredictable mild stress disrupts performance on an object-context mismatch test in female, but not male, mice (Shepard and Coutellier, 2018). Despite this discrepant finding, it is clear that chronic stress can have very different effects on the structure and function of mPFC in males versus females.

Together, these studies suggest that male rats are susceptible to the immediate deleterious effects of chronic stress on the structure and function of mPFC. In contrast, female rats may have some degree of resistance to chronic stress. This pattern is paradoxical, given the female-biased prevalence of stress-linked psychopathologies. However, it is possible that the lack of stress-induced changes in female rats may not be 'resistance', but instead may represent a lack of adaptation to stress. Stressful life events have well-documented effects on the brain and behavior in individuals who do not go on to develop stress-related psychological disorders, suggesting there are sequelae of post-stress changes that should be expected as the healthy brain adapts to stressors. Therefore, if the lack of changes in female rats does represent poor stress adaptation, this may confer some degree of risk for deleterious effects of future stressors. Thus, it is important to investigate sex differences in the lasting effects of stress on the healthy adult brain.

Long-Term Sequelae of Chronic Stress in Rodents

Very few rodent studies have examined the lasting effects of chronic stress in adulthood on brain and behavior. Studies in male rats suggest that the effects of stress are somewhat reversible. For example, much like the effects of chronic stress on dendritic morphology in mPFC, dendritic atrophy also occurs in hippocampal CA3 pyramidal neurons following 21 days of stress (Conrad et al., 1999), which is associated with deficits in spatial working memory (Conrad et al., 1996). However, dendritic retraction is ameliorated in male rats given a 10 day post-stress rest period (Conrad et al., 1999), which is accompanied by behavioral recovery (Sousa et al., 2000). Likewise, chronic stress-induced dendritic retraction is reversed in mPFC following a 21 day rest period (Radley et al., 2005), although it is currently unknown whether chronic stress-induced behavioral deficits are also ameliorated following a rest period.

Despite the reversibility of stress-induced dendritic retraction, there is some evidence that mPFC of male rats is functionally distinct from that of stress-naïve male rats. For example, novel

stress-induced c-*fos* mRNA expression is blunted in male rats up to 30 days after the cessation of chronic variable stress compared to rats exposed only to acute stress (Ostrander et al., 2009). That mPFC of chronically stressed male rats has an altered response to novel stress even after a rest period, combined with dendritic reorganization during this time, suggests that while the immediate effects of stress on mPFC are not permanent, a return to pre-stress function is unlikely. Instead, stress leaves a lasting impact on how mPFC of male rats functions, likely with important, yet currently unknown, behavioral ramifications during this post-stress period.

The most striking evidence for the emergence of post-stress adaptations during a rest period, as opposed to a reversal of stress-induced changes, comes from Gray and colleagues (2014), who examined gene expression in hippocampus of male mice following acute stress, chronic stress, the combination of the two, and acute stress following a post-chronic stress rest period. They found that there was surprisingly little overlap in the genes that were up- and downregulated immediately following chronic stress versus following chronic stress and a rest period. That is, the genes that were upregulated following chronic stress were not the genes that were subsequently downregulated following a rest period. This suggests that while chronic stress and chronic stress followed by a rest period both result in large-scale changes in gene expression in hippocampus of male mice, the pattern of these changes is markedly different. This raises the possibility that the response to another stressor during the extended period following chronic stress could be categorically different from the response of a stress-naïve animal. Indeed, when exposure to a novel acute stressor occurred following a post-chronic stress rest period, the pattern of gene expression changes was strikingly different from that of mice exposed only to the acute stressor (Gray et al., 2014). This is likely due to the recruitment of distinct stress adaptation mechanisms, first to cope with chronic stress, and then to potentially prepare an organism for exposure to future stressors.

Much less is known about post-chronic stress rest periods in female rats. McFadden and colleagues (2011) demonstrated that chronic stress-induced facilitation spatial learning in female

rats in the Morris water maze, a hippocampus-dependent task, is maintained following a 21-day no-stress period. A similar study found that while female rats had no behavioral alterations immediately after chronic stress, following a 21-day rest period, chronically stressed females performed more poorly in the Morris water maze than did unstressed females (Ortiz et al., 2015), suggesting behavioral changes can emerge following a no-stress rest period. What is currently unknown, and will be assessed in this dissertation, is whether a similar pattern of results emerges using a PFC-dependent task.

Summary

In sum, dysfunction of prefrontal cortex is an important part of the pathophysiology of stress-linked disorders (Fossati et al., 2002), and contributes to deficits observed in many cognitive domains, including cognitive flexibility and working memory (Rock et al., 2014). Many of these stress-related disorders are more prevalent in women than men (Cover et al., 2014), although the mechanisms underlying this discrepancy are poorly understood. Studies using rodent models show that male rats are susceptible to the effects of chronic stress in that there are numerous neurobiological and behavioral changes observed shortly after the cessation of stress (Holmes and Wellman, 2009). In contrast, female rats appear to be resilient to the effects of chronic stress on mPFC structure and function, a paradoxical finding given data from clinical studies of stress-related disorders. Despite abundant research on the immediate effects of stress on mPFC and mPFC-mediated behaviors, little is known about the post-stress changes in mPFC that may result in sex differences in responsivity to future stressors. Therefore, my dissertation research examines sex differences in the structure and function of mPFC in the extended period post-chronic stress, and potential sex differences in the effects of a subsequent novel stressor.

Organization of the Dissertation

This thesis is divided into 6 chapters. This first chapter provides a review of the relevant literature on sex differences in the immediate and lasting effects of stress on the brain and behavior that informed the experiments that follow. Chapter 2 is the first experimental chapter, in which I examine sex differences in dendritic remodeling in the prelimbic subregion of mPFC immediately following chronic stress, as well as following 7- and 10-day rest periods. In Chapter 3, I examine if the sex-specific pattern of dendritic reorganization during the post-stress rest period found in Chapter 2 corresponds to sex differences in neuronal activation in response to a novel acute stressor during this time. In addition to prelimbic cortex, I also examine other corticolimbic brain regions to begin to characterize circuit-level effects of chronic stress on novel stress-induced neuronal activation. Here, I show that chronically stressed male, but not female, rats have a persistent reduction in novel stress-induced neuronal activation in prelimbic cortex. In Chapter 4, I examine whether there are similar sex-specific changes in extradimensional set-shifting, a task mediated by mPFC. In Chapter 5, I begin to investigate potential mechanisms that may contribute to sex-specific changes in the function of prelimbic cortex during the post-chronic stress period. Specifically, I assess changes in the expression of genes relevant to glutamatergic and GABAergic neurotransmission following chronic and two-hit stress. All experimental chapters include a specific introduction with a rationale for the experiment, a methods section, results with relevant figures, and a discussion of the results from that experiment. A general discussion is provided in Chapter 6 in which I synthesize and interpret the major findings from the dissertation and discuss the broader implications of these findings in the study of sex differences in the effects of stress and post-stress rest periods on mPFC.

Chapter 2:

Differential dendritic remodeling in prelimbic cortex of male and female rats during recovery from chronic stress.

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Both acute and chronic stress profoundly alter the morphology of pyramidal neurons in the prelimbic (PL) region of the rodent medial prefrontal cortex (mPFC). In males, acute (Nava et al., 2015), mild (Brown et al., 2005), and chronic stress (Cook and Wellman, 2004; Radley et al., 2004; Liu and Aghajanian, 2008) result in the retraction of apical dendrites on pyramidal neurons. This retraction is coupled with a decrease in spine density following chronic stress (Radley et al., 2006; Radley et al., 2008), but an increase in spine density after acute stress (Nava et al., 2015).

Although the morphology of pyramidal neurons in mPFC can undergo rapid and robust changes in response to stress, these changes are reversible in males. For instance, dendritic retraction in PL following chronic stress is ameliorated after a 21 day post-stress rest period (Radley et al., 2005; Bloss et al., 2010). There is also evidence that a shorter length of time may be sufficient for this process to occur. For example, retraction of apical dendrites on pyramidal neurons in hippocampus subfield CA3 of male occurs following chronic stress, but this retraction is absent following just a 10 day post-stress rest period (Conrad et al., 1999). Therefore, it is possible that a shorter post-stress rest period may be sufficient for the amelioration of dendritic retraction in PL following chronic stress in males.

In contrast to the dendritic retraction following chronic stress observed in males, apical dendritic outgrowth occurs on pyramidal neurons in PL of female rats (Garrett and Wellman, 2009). It is currently unknown whether this outgrowth is reversible. Therefore, to further characterize the process of dendritic reorganization in mPFC following chronic stress, I assessed dendritic morphology in PL of male and female rats immediately following chronic restraint stress, as well as after 7 and 10 day post-stress rest periods.

Materials and Methods

Subjects and Stressors

Male and female Sprague Dawley rats (approximately 68 days of age at start; Harlan, Indianapolis, IN; N = 73) were group-housed (3 per cage) in standard laboratory cages (48 cm × 20 cm × 26 cm), with ambient temperature 23-25 °C, free access to food and water, and a 12:12 light/dark cycle (lights on at 0800 h). Rats were either left unstressed or subjected to chronic restraint stress for 10 days, and were given a recovery (Rec) period of 0, 7, or 10 days, resulting in 8 groups (Fig. 2.1): unstressed males (n = 11) and females (n = 12), 0d Rec males (n = 8) and females (n = 8), 7d Rec males (n = 7) and females (n = 9), and 10d Rec males (n = 9) and females (n = 9). All rats were weighed daily throughout the stress procedure. Immediately after weighing, unstressed rats were returned to their home cages and left undisturbed for 3 hours in a separate room. Stressed rats were placed in semi-cylindrical restrainers (6.35 cm diameter x 15.24 cm length, modified so the tail piece locks into place; Braintree Scientific) for 3 hours in their home cages, a manipulation that produces significant increases in plasma corticosterone levels (Cook and Wellman, 2004). Rats were left undisturbed during the recovery period. All procedures were conducted between 8:00 am and 8:00 pm (i.e., during the light phase), were in accordance with NIH Guidelines, and were approved by the Indiana University's IACUC.

Estrous Phase Characterization

On the day of perfusion, vaginal lavages were performed and exfoliate cytology was examined immediately under light microscopy. Estrous phase was determined based on the morphology of cells present (Garrett and Wellman, 2009). Due to the small number of rats in proestrus (n = 3) and estrus (n = 2) compared to diestrus (n = 33), we did not analyze our data relative to estrous phase.

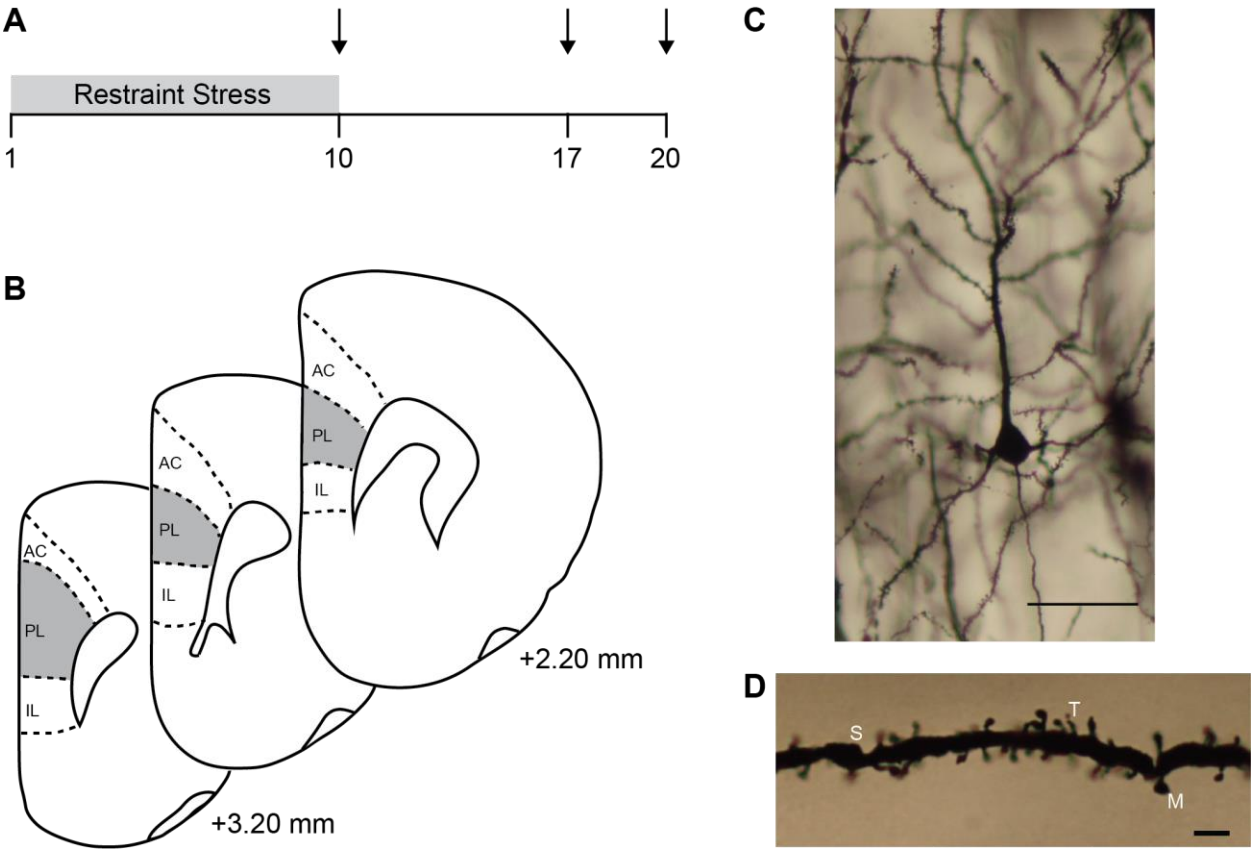


Figure 2.1. (A) Schematic depiction of experimental timeline for assessing dendritic morphology in chronically stressed males and females during a post-stress recovery period. Arrow represents tissue collection. (B) Schematic diagram of coronal sections through rat prefrontal cortex. The portions of prelimbic cortex from which neurons were sampled is shown (shaded areas). Coordinates indicate position relative to bregma (Paxinos and Watson, 1998). (C) Digital micrograph of Golgi-stained neuron in layer II-III of mPFC. Scale bar = 50 µm. (D) Digital

Corticosterone EIA

Immediately prior to perfusion, blood was collected via cardiac puncture and allowed to clot at room temperature for 30 minutes followed by centrifugation at 13,000 rpm for 5 minutes to obtain serum. Corticosterone was measured via a commercially available EIA kit (Enzo Life Sciences, Plymouth Meeting, PA) that shows low crossreactivity with other major steroid hormones. Samples were diluted (1:20) with assay buffer and run in duplicates according to instructions provided by the manufacturer. The sensitivity of the assay was 27 pg/mL, and intra-assay variation was 1.77% and 2.62% for each plate.

Histology and Dendritic Analysis

Brains were processed using a modification of Glaser and van der Loos' Golgi stain, as described previously (Glaser and Van der Loos, 1981; Martin and Wellman, 2011). On the final day of either stress or recovery, rats were deeply anesthetized with urethane and transcardially perfused with saline. To verify the stress manipulation, adrenal glands were removed and weighed. Brains were removed and immersed in Golgi-Cox solution for 14 days and then moved to 30% sucrose in saline (Gibb and Kolb, 1998). Brains were sectioned at 200 µm on a vibratome (Campden Instruments, MA752). Sections were mounted, alkalinized, developed in Dektol (Kodak), fixed in Ilford rapid fixer, dehydrated in a graded series of ethanols, cleared in xylenes, and coverslipped (Wellman, 2016).

Pyramidal neurons in layer II-III of prelimbic cortex were reconstructed (Fig. 2.1 B). Prelimbic cortex is readily identified by its position on the medial wall of the rostral cortex, and its location dorsal to infralimbic cortex, which is markedly thinner and has fewer, less well-defined layers (Zilles and Wree, 1995), and ventral to anterior cingulate cortex, which is thicker than PL and located adjacent to the inflection of the forceps minor, which results in a distinct bend to the apical trunk of its layer II-III neurons . Within PL, layer II-III is readily identifiable in Golgi-stained material based on its characteristic cytoarchitecture. Its position is immediately lateral to the

relatively cell-poor layer I (which also contains the distal dendritic tufts of layer II-III pyramid cells) and medial to layer V. In mPFC, this boundary is pronounced because of the greater cell-packing density and smaller somata of pyramidal cells in layer II-III relative to layer V (Cajal, 1995; Zilles and Wree, 1995). Pyramidal neurons were defined by a distinct, single apical dendritic tree extending from the apex of the soma toward the pial surface of the cortex, two or more basilar dendritic trees extending from the base of the soma, and the presence of dendritic spines (Fig. 2.1 C). Neurons selected for reconstruction did not have truncated apical branches, had at least one nontruncated basilar tree, and were unobscured by neighboring neurons and glia, with dendrites that were easily discriminable by focusing through the depth of the tissue. In 4 sections evenly spaced through the rostral-caudal extent of PL, all pyramidal neurons meeting these criteria were identified (mean = 24 ± 6 per animal). Eight neurons per animal (four from each hemisphere) were then randomly selected from all of those identified and reconstructed at a final magnification of 600x. Morphology of apical and basilar arbors was quantified in three dimensions using a computer-based neuron tracing system (Neurolucida, MBF Biosciences) with the experimenter blind to condition. For basilar dendrites, only non-truncated basilar trees were drawn, and per-tree analyses were performed.

Spines were also counted on eight neurons per animal. For each neuron, a segment averaging 39.37 ± 0.35 µm in length was drawn from one terminal apical and one terminal basilar dendrite. Whenever possible, spines were assessed on previously reconstructed neurons. We examined distal branches because stress and corticosterone administration induce dendritic remodeling in these branches (Wellman, 2001; Cook and Wellman, 2004; Liu and Aghajanian, 2008). Spines were classified as stubby, thin, or mushroom, based on standard morphological criteria (Fig. 2.1 D; Peters and Kaiserman-Abramof, 1970).

Statistical analyses

Chronic stressors such as immobilization and restraint attenuate normal weight gain in males (Marti et al., 1994; Cook and Wellman, 2004) and increase relative adrenal weight (Heiderstadt et al., 2000). Therefore, to verify the stress manipulation, weight gain and adrenal weight were compared using two-way ANOVAs (stress x sex). To examine possible differences in lasting basal hypothalamic-pituitary-adrenal (HPA) axis activity as a result of chronic stress, mean serum corticosterone was compared using a two-way ANOVA (stress x sex).

To assess changes in the amount and distribution of dendritic material, a three-dimensional version of a Sholl analysis (Sholl, 1956) was used, in which the total length of dendrites between an overlay of concentric spheres of 10 μm radius centered on the soma was assessed. To simplify analyses, the lengths of apical and basilar arbors were divided into thirds (proximal, middle, and distal) based on the cumulative lengths of unstressed males and females. These data were compared across groups using three-way repeated measures ANOVAs (distance from soma x stress x sex). Total spine density and densities of each spine type were also compared using two-way ANOVAs (stress x sex). We also estimated the total number of spines, and the total number of each spine type, on terminal branches by multiplying spine density by terminal branch length. These data were compared using two-way ANOVAs (stress x sex). For all analyses, measures were averaged across neurons within each animal; thus, the unit of analysis for statistics is the individual animal. Planned comparisons were done when appropriate, and consisted of F tests done within the context of the overall ANOVA (Hayes, 1994; Maxwell and Delaney, 2004), comparing unstressed rats to stressed rats given 0, 7, or 10 days to recover and comparing each recovery group to the 0 day recovery rats.

Results

Body and Adrenal Weight Analyses

Weight change differed between males and females (Fig. 2.2 A; main effect of sex, $F_{1,65} = 36.32$, $p < 0.05$). Further, chronic stress and subsequent recovery significantly altered weight change in males and females (main effect of stress, $F_{3,65} = 47.21$, $p < 0.05$), although this effect was different in males and females (sex x stress interaction, $F_{3,65} = 13.05$, $p < 0.05$). Planned comparisons revealed that 0d Rec males showed significant weight attenuation compared to unstressed males ($F_{1,17} = 72.89$, $p < 0.001$). Further, 7d Rec males gained significantly more weight than unstressed males ($F_{1,18} = 5.05$, $p = 0.04$). A similar trend approached significance in 10d Rec males ($F_{1,16} = 4.08$, $p = 0.06$). Further, both 7d and 10d Rec males gained significantly more weight than 0d Rec males (7d Rec, $F_{1,15} = 71.04$, $p < 0.001$; 10d Rec, $F_{1,13} = 56.99$, $p < 0.001$), although these two groups did not differ from each other ($F_{1,14} = 0.01$, ns).

Chronic stress did not significantly attenuate weight gain in females (unstressed vs 0d Rec, $F_{1,18} = 2.71$, ns). However, increased weight gain in 7d Rec females tended toward significance compared to unstressed females ($F_{1,19} = 4.11$, $p = 0.06$), and was significantly greater compared to 0d Rec ($F_{1,15} = 14.07$, $p = 0.002$). Ten day recovery females gained significantly more weight than both unstressed ($F_{1,19} = 10.59$, $p = 0.004$) and 0d Rec ($F_{1,15} = 23.74$, $p < 0.001$) females. Females in the 7d and 10d rec groups did not differ from each other ($F_{1,16} = 2.20$, ns).

In agreement with previous work (Konkle et al., 2003), females had a higher adrenal-to-body weight ratio (Fig. 2.2 B; main effect of sex, $F_{1,65} = 366.26$, $p < 0.05$). Further, chronic stress, as well as the presence of a recovery period, significantly altered adrenal weight in both males and females (main effect of stress, $F_{3,65} = 16.82$, $p < 0.05$). Planned comparisons revealed that chronic stress increased adrenal weight relative to body weight in both 0d Rec males ($F_{1,17} = 30.34$, $p < 0.001$) and 0d Rec females ($F_{1,18} = 13.38$, $p = 0.002$), while 7d and 10d Rec males and females did not differ from same-sex controls (all $Fs \leq 1.38$, all ns). On the other hand, 7d and 10d Rec males and females had significantly lower relative adrenal weights relative compared to

stressed males and females that had no recovery period (all *F*s ≥ 10.71, all *p*s ≤ 0.006). Adrenal weights did not differ between 7day and 10d males ($F_{1,14}$ = 1.07, ns) or females ($F_{1,16}$ = 0.03, ns).

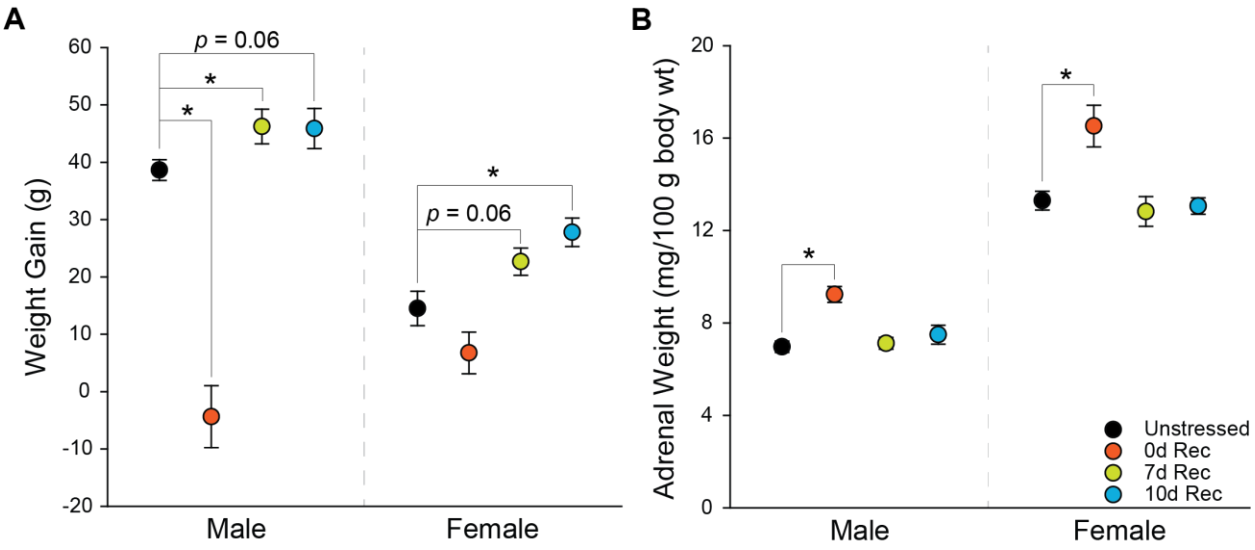


Figure 2.2. (A) Mean weight change in unstressed versus stressed male and female rats given a 0d, 7d, or 10d recovery. Stress attenuated weight gain in 0d Rec male and female rats. (B) Mean adrenal-weight-to-body-weight ratios (adrenal weight/100 g body weight) in unstressed versus stressed male and female rats receiving a 0d, 7d, or 10d recovery. Stress increased relative adrenal weight in 0d Rec male and female rats. Error bars represent SEM. * p < 0.05.

Corticosterone Analysis

Baseline corticosterone levels differed between males and females (Fig. 2.3; main effect of sex, $F_{1,65}$ = 13.14, p < 0.05). Although there was no main effect of stress ($F_{3,65}$ = 1.33, ns), there was a significant sex by stress interaction ($F_{3,65}$ = 2.71, p = 0.05). Planned comparisons revealed that at all recovery time points, stressed females had higher serum corticosterone concentrations than unstressed females (0d Rec, $F_{1,18}$ = 4.32, p = 0.05; 7d Rec, $F_{1,19}$ = 5.24, p = 0.03; 10d Rec, $F_{1,19}$ = 10.28, p = 0.005), while 0d, 7d, and 10d Rec females did not differ from each other (all *F*s ≤ 0.76, all ns). Female 7d and 10d Rec groups had significantly higher corticosterone than males

at the same recovery time points (7d, $F_{1,16} = 6.49$, $p = 0.02$; 10d, $F_{1,14} = 8.92$, $p = 0.01$). There were no significant differences in baseline corticosterone levels between unstressed males and stressed males at any recovery point, although there was a trend for decreased corticosterone in the 7d Rec group (0 days, $F_{1,17} = 1.34$, ns; 7 Days, $F_{1,18} = 3.53$, $p = 0.08$; 10 days, $F_{1,16} = 2.78$, ns).

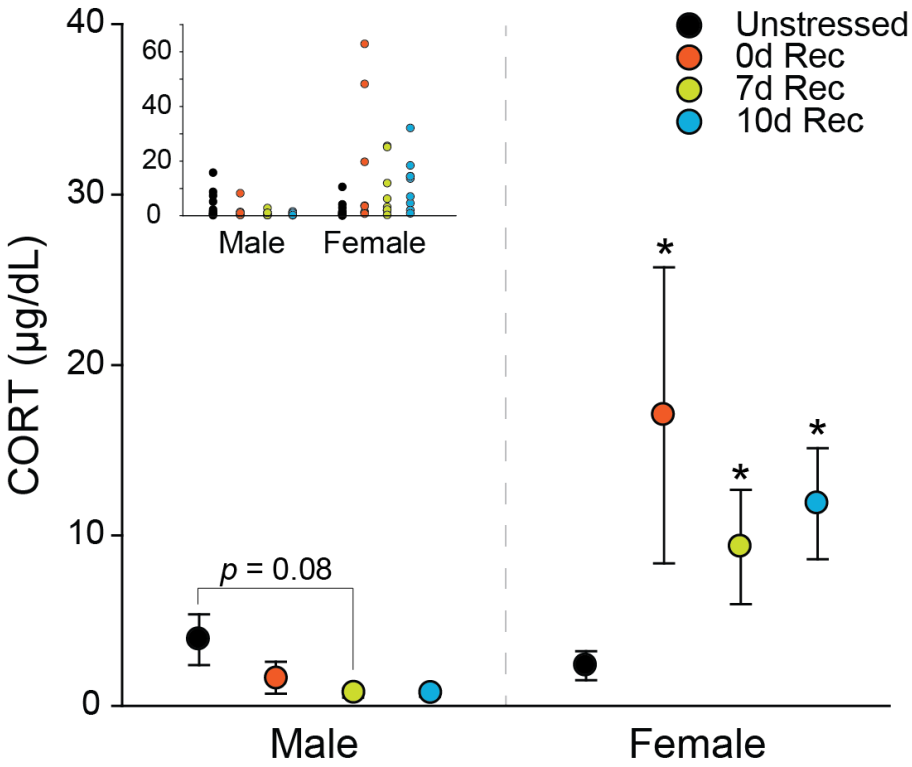


Figure 2.3. Mean serum CORT at perfusion in unstressed versus stress male and female rats receiving a 0d, 7d, or 10d recovery. While 7d Rec male rats tended to have suppressed baseline CORT, stressed females had elevated CORT at all times post-stress. Error bars represent SEM. Inset: Scatterplot of individual datapoints. Error bars represent SEM. * $p < 0.05$.

Dendritic Analyses

Stress significantly altered apical dendritic length ($F_{3,65} = 6.25$, $p < 0.05$). Although there was no main effect of sex ($F_{1,65} = 2.58$, ns) or distance from soma ($F_{1,65} = 0.03$, ns) and no interaction between sex and stress ($F_{3,65} = 0.13$, ns), there were significant interactions between distance from the soma and stress ($F_{3,65} = 2.56$, $p < 0.05$), as well as distance from the soma and sex ($F_{2,130} = 3.37$, $p < 0.05$). The three-way interaction between distance from the soma, sex, and stress was non-significant ($F_{6,130} = 0.65$, ns), and therefore, two-way ANOVAs (stress x distance from soma) were used to examine the effects of stress on apical branch length separately in males and females.

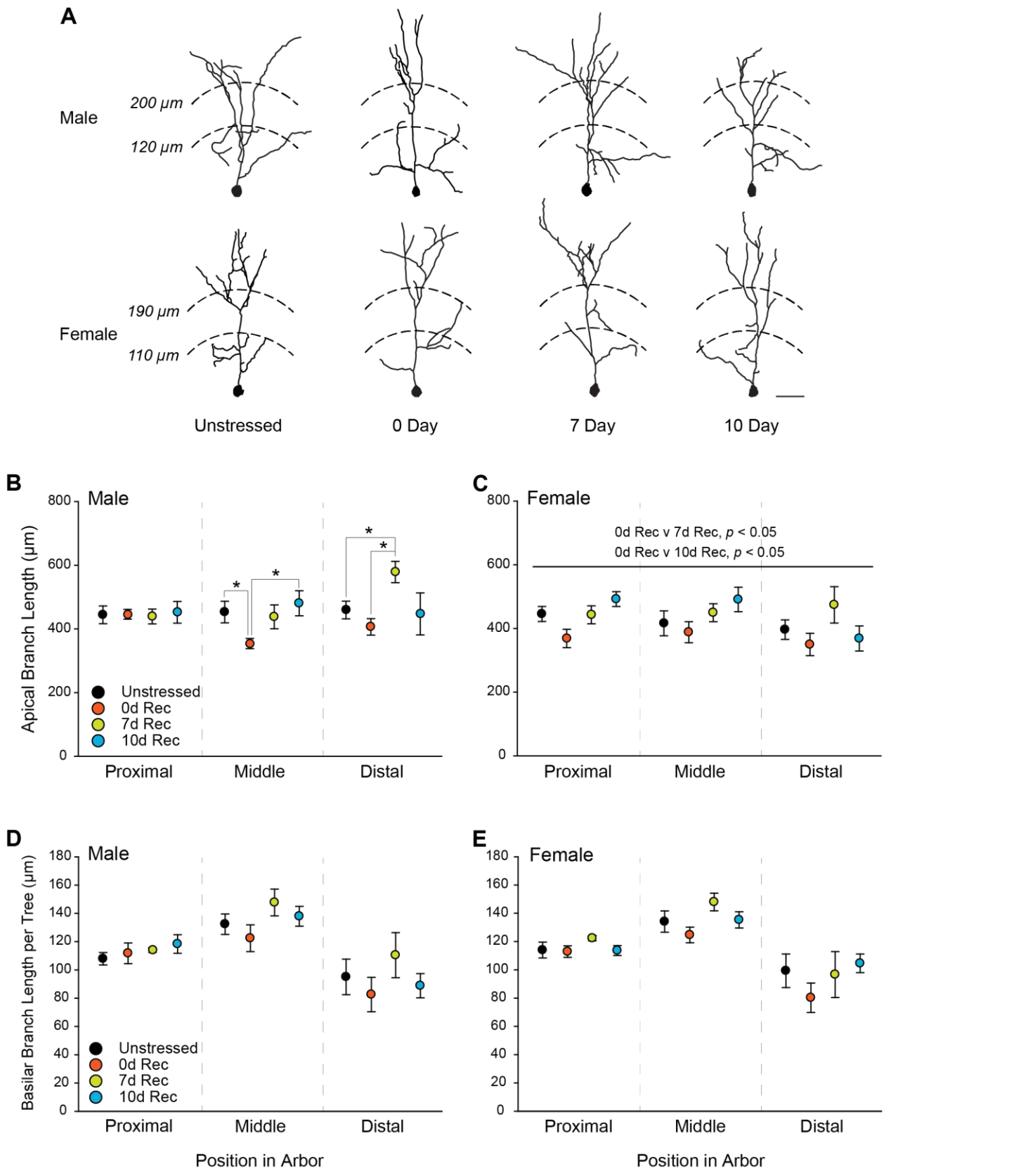
In males, stress tended to alter apical branch length ($F_{3,31} = 2.73$, $p = 0.06$). While there was no main effect of distance from the soma ($F_{2,62} = 1.80$, ns), apical dendritic length varied across stress conditions as a function of distance from the soma (Fig. 2.4 B; distance from soma x stress interaction, $F_{6,62} = 2.37$, $p < 0.05$). Planned comparisons revealed no significant differences proximal to the cell body (all Fs ≤ 0, all ns). In contrast, dendritic length in the middle third of the apical arbor was significantly reduced in 0d Rec males compared to unstressed males ($F_{1,17} = 5.50$, $p = 0.03$). Further, a trend towards increased dendritic length in 7d Rec males compared to 0d Rec males approached significance ($F_{1,15} = 3.86$, $p = 0.07$) and reached significance in 10d Rec males ($F_{1,13} = 9.75$, $p = 0.008$). Surprisingly, in the distal portion of the apical arbor, branch length in 7d Rec males was significantly greater than that of either unstressed males ($F_{1,18} = 7.66$, $p = 0.01$) or 0d Rec males ($F_{1,15} = 16.05$, $p = 0.001$), and this difference approached significance compared to 10d Rec males ($F_{1,14} = 3.64$, $p = 0.08$). All other planned comparisons were non-significant (all Fs ≤ 0.84, all ns).

Apical dendritic reorganization was also seen in females (Fig. 2.4 C; main effect of stress, $F_{3,34} = 3.7$, $p < 0.05$). This effect did not vary across the dendritic arbor (main effect of distance from soma, $F_{2,68} = 1.66$, ns; distance from soma x stress interaction, $F_{6,68} = 1.08$, ns). Therefore, follow-up comparisons were performed on total apical branch length, collapsed across the arbor.

A small decrease in apical branch length in 0d Rec females compared to unstressed females approached significance ($F_{1,18} = 3.66$, $p = 0.07$). Further, 7d Rec and 10d Rec females had greater branch length than 0d Rec females (7d, $F_{1,15} = 7.50$, $p = 0.02$; 10d, $F_{1,15} = 7.14$, $p = 0.02$). Apical branch length was not different in 7d and 10d Rec relative to unstressed females (all Fs ≤ 0.77, ns).

Consistent with prior work (e.g., Cook and Wellman, 2004; Brown et al., 2005; Izquierdo et al., 2006), basilar dendritic length varied by distance from soma ($F_{2,130} = 2.37$, $p < 0.05$). However, there was no main effect of stress ($F_{3,65} = 2.14$, ns) or sex ($F_{1,65} = 0.11$, ns) on basilar branch length (Fig. 2.4 D, E), and no stress by sex interaction ($F_{3,65} = 0.94$, ns).

Figure 2.4. (A) Computer-assisted reconstruction of apical arbors of Golgi-stained neurons in layer II-III of PL in unstressed, 0d, 7d, and 10d Rec male and female rats. Neurons are at or near the mean for each group. Scale bar = 50 µm. (B) Mean length of apical branches in unstressed, 0d, 7d, and 10d Rec male rats with 10-µm concentric circles summed across the proximal, middle, and distal third of the arbor. 0d Rec male rats have decreased dendritic length in the middle third of the arbor, while 7d Rec male rats have increased dendritic length in the distal third. (C) Mean length of apical branches in unstressed, 0d, 7d, and 10d Rec females with 10-µm concentric circles summed across the proximal, middle, and distal third of the arbor. Stressed females have no changes in dendritic length across the apical arbor at any time post-stress. (A) Mean length of basilar branches per tree in unstressed, 0d, 7d, and 10d Rec male rats with 10-µm concentric circles summed across the proximal, middle, and distal third of the arbor. (B) Mean length of basilar branches per tree in unstressed, 0d, 7d, and 10d Rec female rats with 10-µm concentric circles summed across the proximal, middle, and distal third of the arbor. Overall basilar branch length did not vary across stress condition or sex. Error bars represent SEM. * $p < 0.05$


A
Male
200 µm
120 µm
Female
190 µm
110 µm
Unstressed
0 Day
7 Day
10 Day
B
Male
Apical Branch Length (µm)
800
600
400
200
0
Unstressed
0d Rec
7d Rec
10d Rec
Proximal
Middle
Distal
*
C
Female
0d Rec v 7d Rec, p < 0.05
0d Rec v 10d Rec, p < 0.05
D
Male
Basilar Branch Length per Tree (µm)
180
160
140
120
100
80
60
40
20
0
Position in Arbor
E
Female

Apical Spine Density

Total apical spine density, as well as the densities of apical thin and mushroom spines were unaffected by stress (Fig 2.5 A, C, D; all *F*s ≤ 1.87, all ns), did not differ between males and females (all *F*s ≤ 0.96, all ns), and there were no significant interactions (all *F*s ≤ 1.25, all ns). In contrast, despite no main effects of stress ($F_{3,65}$ = 0.93, ns) or sex ($F_{1, 65}$ = 1.25, ns) on stubby spine density, stress altered stubby spine density differentially in males and females (Fig. 2.5 B; for sex x stress interaction, $F_{3,65}$ = 3.72, p < 0.05). Planned comparisons revealed that whereas 0d Rec males had a decrease in stubby spine density relative to unstressed males ($F_{1,17}$ = 4.31, p = 0.05), 7d Rec females showed an increase in stubby spine density relative to unstressed females. ($F_{1,19}$ = 5.77, p = 0.03). This pattern of changes resulted in a significant sex difference in stubby spine density immediately following chronic stress ($F_{1,14}$ = 5.38, p = 0.04), whereby males had a significantly lower stubby spine density compared to females. A similar, although non-significant, trend was found at 7 days post-stress ($F_{1,16}$ = 3.75, p = 0.07). No other planned comparisons reached significance (all *F*s ≤ 2.40, all ns).

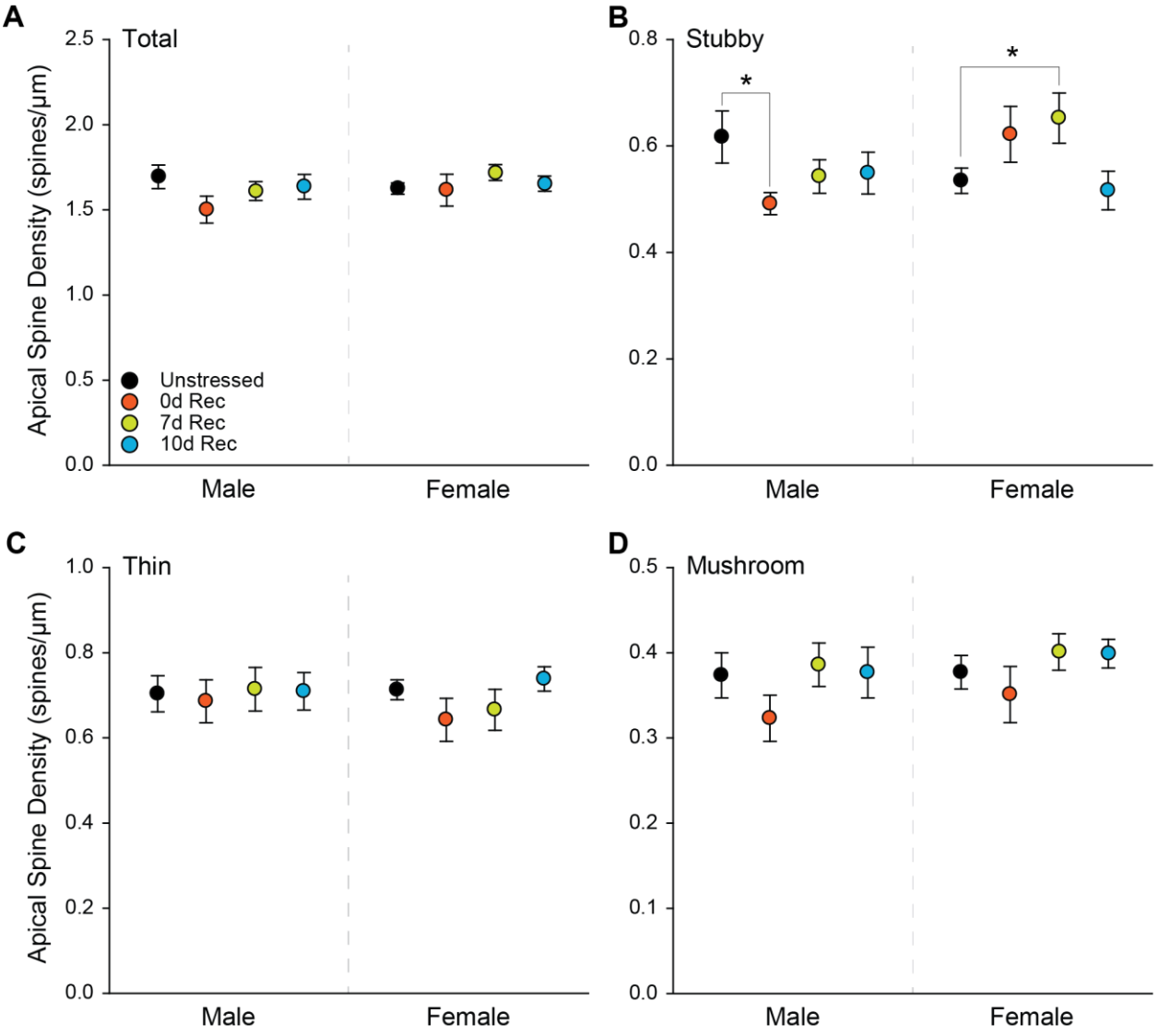


Figure 2.5. (A) Total apical spine density in unstressed, 0d, 7d, and 10d Rec male and female rats. (B) Apical stubby spine density in unstressed, 0d, 7d, and 10d Rec male and female rats. Chronic stress decreased stubby spine density in 0d Rec male rats, but increase spine density in 7d Rec females. (C) Apical thin spine density in unstressed, 0d, 7d, and 10d Rec male and female rats. (D) Apical mushroom spine density in unstressed, 0d, 7d, and 10d Rec male and female rats. Error bars represent SEM. * $p < 0.05$.

Estimated Apical Terminal Spines

Estimation of terminal spines revealed a different pattern of stress-induced changes on apical dendritic spines. Stress altered the estimated number of total (Fig. 2.6 A; $F_{3,65} = 4.20$, $p < 0.05$), thin (Fig. 2.6 C; $F_{3,65} = 3.23$, $p < 0.05$), and mushroom (Fig. 2.6 D; $F_{3,65} = 3.92$, $p < 0.05$) spines. These effects did not vary by sex (All Fs < 0.82, all ns), and there were no significant stress by sex interactions (All Fs < 0.53, all ns). Planned comparisons revealed 0d Rec rats had fewer estimated total spines than unstressed ($F_{1,37} = 6.58$, $p = 0.01$), 7d Rec ($F_{1,32} = 11.37$, $p = 0.002$), and 10d Rec rats ($F_{1,30} = 9.62$, $p = 0.004$). A similar pattern was found for estimated thin (unstressed, $F_{1,37} = 5.73$, $p = 0.02$; 7d Rec, $F_{1,32} = 5.73$, $p = 0.02$; 10d Rec, $F_{1,30} = 10.35$, $p = 0.003$) and mushroom (unstressed, $F_{1,37} = 5.86$, $p = 0.02$; 7d Rec, $F_{1,32} = 11.426$, $p = 0.002$; 10d Rec, $F_{1,30} = 10.63$, $p = 0.003$) spines. For apical stubby spines, although there was no main effect of stress ($F_{3,65} = 2.25$, ns) or sex ($F1_{,65} = .003$, ns), there was a significant stress by sex interaction ($F_{3,65} = 2.69$, $p = 0.05$). Planned comparisons revealed a decrease in estimated apical stubby spines in 0d Rec males compared to unstressed ($F_{1,17} = 6.46$, $p = 0.02$) and 10d Rec males ($F_{1,13} = 5.31$, $p = 0.04$), a pattern that approached significance compared to 7d Rec males ($F_{1,15} = 4.12$, $p = 0.06$). In contrast, 7d Rec female rats had an increased estimated number of stubby spines compared to unstressed females ($F_{1,19} = 9.63$, $p = 0.006$), which tended also to be the case compared to 10d Rec female rats ($F_{1,16} = 4.18$, $p = 0.06$). No other comparisons reached significance (all Fs < 3.70, all ns).

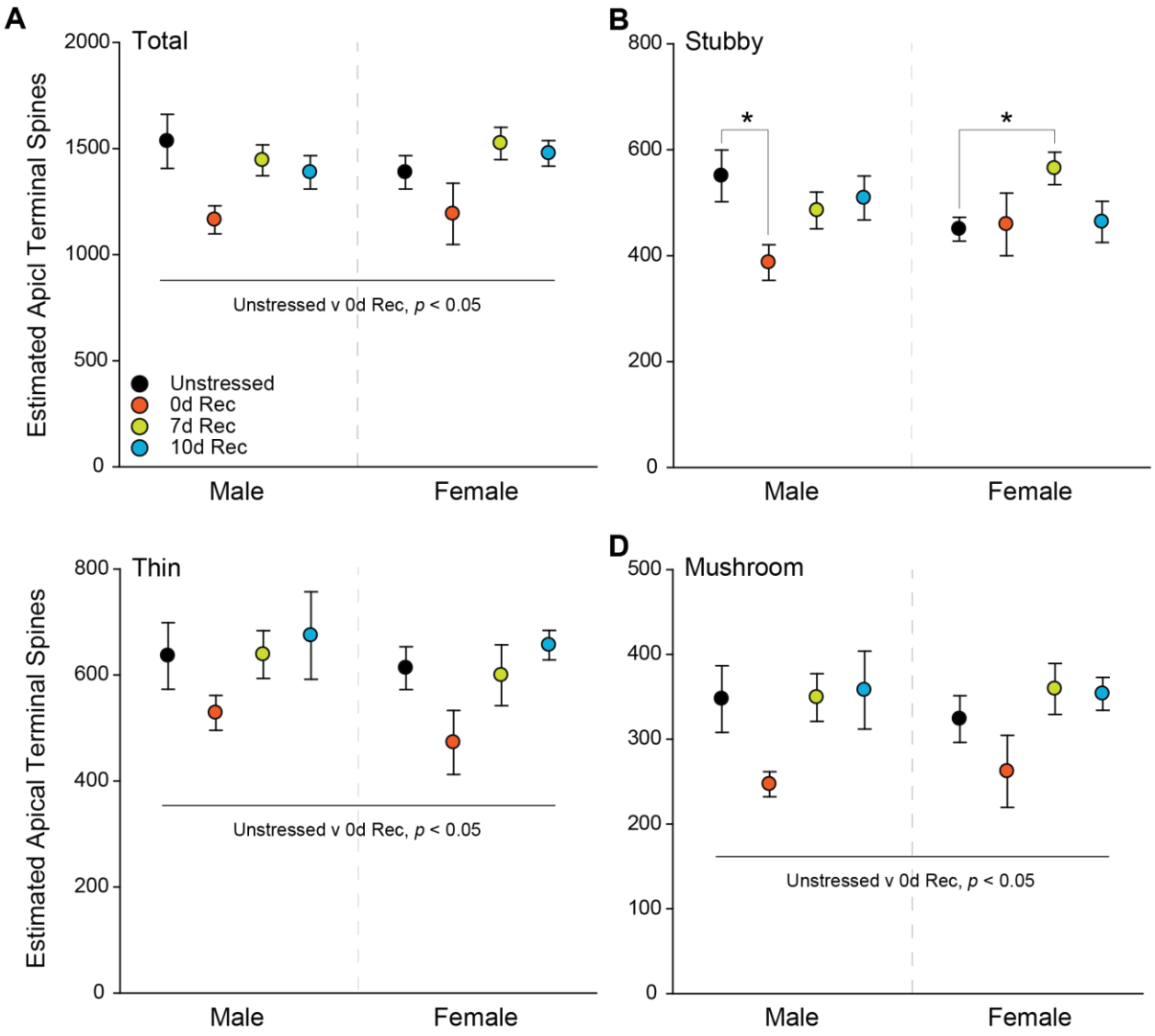


Figure 2.6. Estimated number of total, stubby, thin, and mushroom spines on apical terminal dendrites in unstressed 0d, 7d, and 10d Rec male and female rats. (A) Chronic stress decreased the total number of terminal spines in both male and female 0d Rec rats compared to unstressed rats. (B) In males, chronic stress decreased estimated stubby spine number in 0d Rec rats compared to both unstressed and 10d Rec rats, and this decrease approached significance compared to 7d Rec rats. (C) Chronic stress decreased the estimated number of thin spines in both male and female 0d Rec rats compared to unstressed rats. (D) Chronic stress decreased the estimated number of mushroom spines in both male and female 0d Rec rats compared to unstressed rats. Error bars represent SEM. * $p < 0.05$.

Basilar Spine Density

Significant changes in basilar spines were also observed (Fig. 2.7). While there was no main effect of stress or sex on total basilar spine density, there was a significant stress by sex interaction ($F_{3,65}$ = 3.54, p < 0.05). Planned comparisons revealed that the 0d Rec male group tended to have a decrease in spine density ($F_{1,17}$ = 3.75, p = 0.07). This decrease was significant in 7d Rec males ($F_{1,18}$ = 8.28, p = 0.01). In contrast, no comparisons reached significance in females (all Fs ≤ 0.99, all ns), although the decrease in overall spine density in 7d Rec males resulted in a significant sex difference at this time point ($F_{1,16}$ = 7.95, p = 0.01).

For densities of stubby and thin spines, there were no main effects of stress (Fig. 2.7 B, C; stubby, $F_{3,65}$ = 1.05, ns; thin, $F_{3,65}$ = 0.93, ns) or sex (stubby, $F_{1,65}$ = 0.69, ns; thin, $F_{1,65}$ = 0.02, ns), and no significant interactions (stubby, $F_{3,65}$ = 1.81, ns; thin, $F_{3,65}$ = 1.37, ns). In contrast, stress significantly altered the density of mushroom spines (Fig. 2.7 D; $F_{3,65}$ = 4.55, p < 0.05), and this effect did not differ between males and females (main effect of sex, $F_{1,65}$ = 0.73, ns; stress x sex interaction, $F_{3,65}$ = 0.44, ns). Planned comparisons revealed that 0d Rec rats had significantly decreased density of mushroom spines ($F_{1,37}$ = 5.94, p = 0.02), a pattern that approached significance in 7d Rec rats ($F_{1,37}$ = 3.52, p = 0.07). Further, 10d Rec rats had significantly greater density of mushroom spines compared to either 0d Rec ($F_{1,30}$ = 11.39, p = 0.002) or 7d Rec ($F_{1,30}$ = 7.52, p = 0.01) rats. No other comparisons reached significance (all Fs ≤ 0.74, all ns).

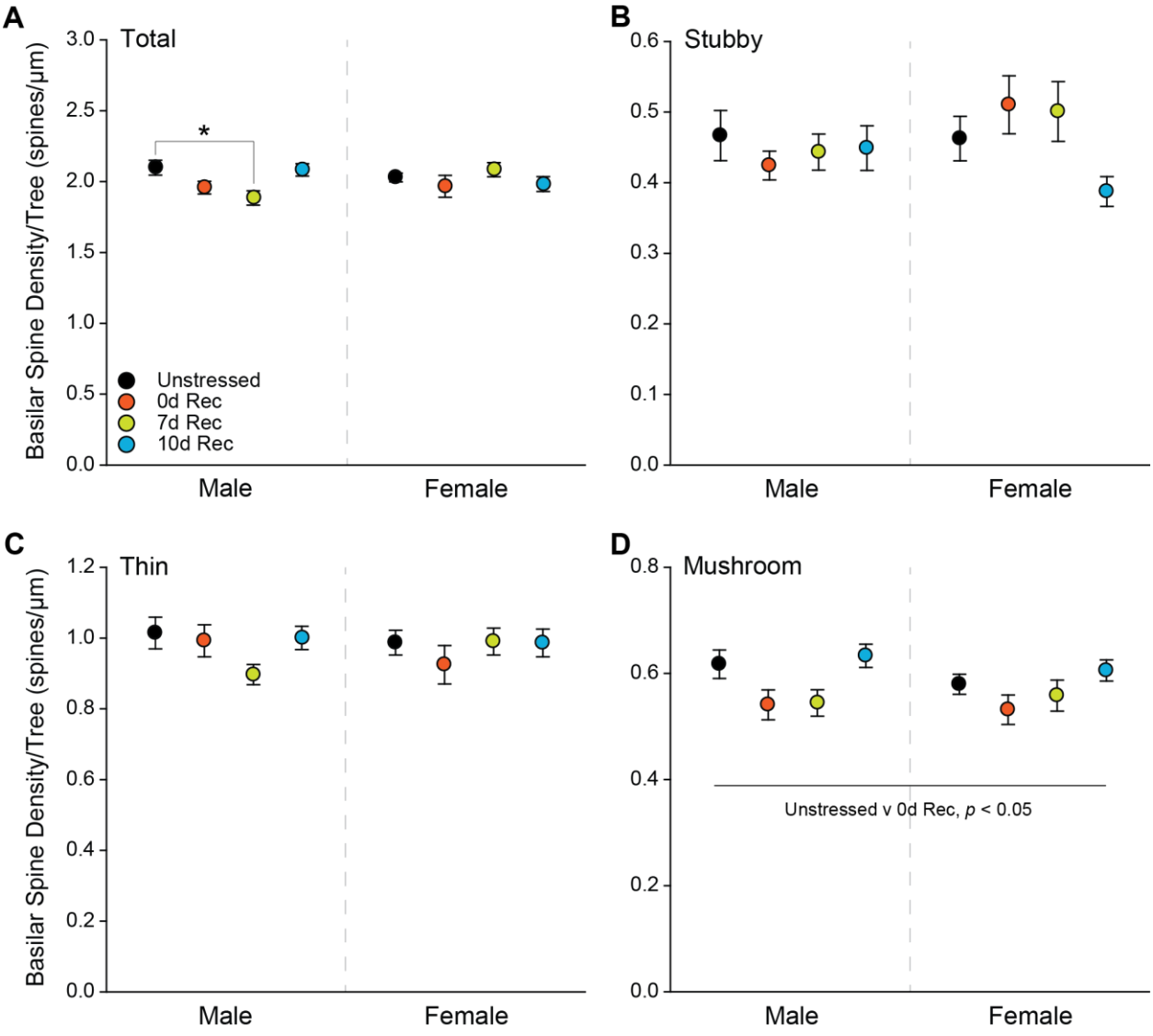


Figure 2.7. (A) Total basilar spine density in unstressed, 0d, 7d, and 10d Rec male and female rats. Chronic stress decreased basilar spine density in 0d and 7d Rec male rats. (B) Basilar stubby spine density in unstressed, 0d, 7d, and 10d Rec male and female rats. (C) Basilar thin spine density in unstressed, 0d, 7d, and 10d Rec male and female rats. (D) Basilar mushroom spine density in unstressed, 0d, 7d, and 10d Rec male and female rats. Chronic stress decrease mushroom spine density in 0d and 7d Rec rats. Error bars represent SEM. * $p < 0.05$.

Estimated Basilar Terminal Spines

The estimated total number of basilar spines did not differ by stress condition ($F_{3,65}$ = 0.74, ns) or sex ($F_{1,65}$ = 0.71, ns), and there was no stress by sex interaction ($F_{3,65}$ = 0.40, ns). This was also the case for the estimated number of stubby (main effect of stress, $F_{3,65}$ = 0.42, ns; main effect of sex, $F_{1,65}$ = 0.39, ns; stress x sex, $F_{3,65}$ = 0.33, ns), thin (main effect of stress, $F_{3,65}$ = 0.75, ns; main effect of sex, $F_{1,65}$ = 0.69, ns; stress x sex, $F_{3,65}$ = 0.44, ns), and mushroom (main effect of stress, $F_{3,65}$ = 1.32, ns; main effect of sex, $F_{1,65}$ = 0.97, ns; stress x sex, $F_{3,65}$ = 0.28, ns) spines.

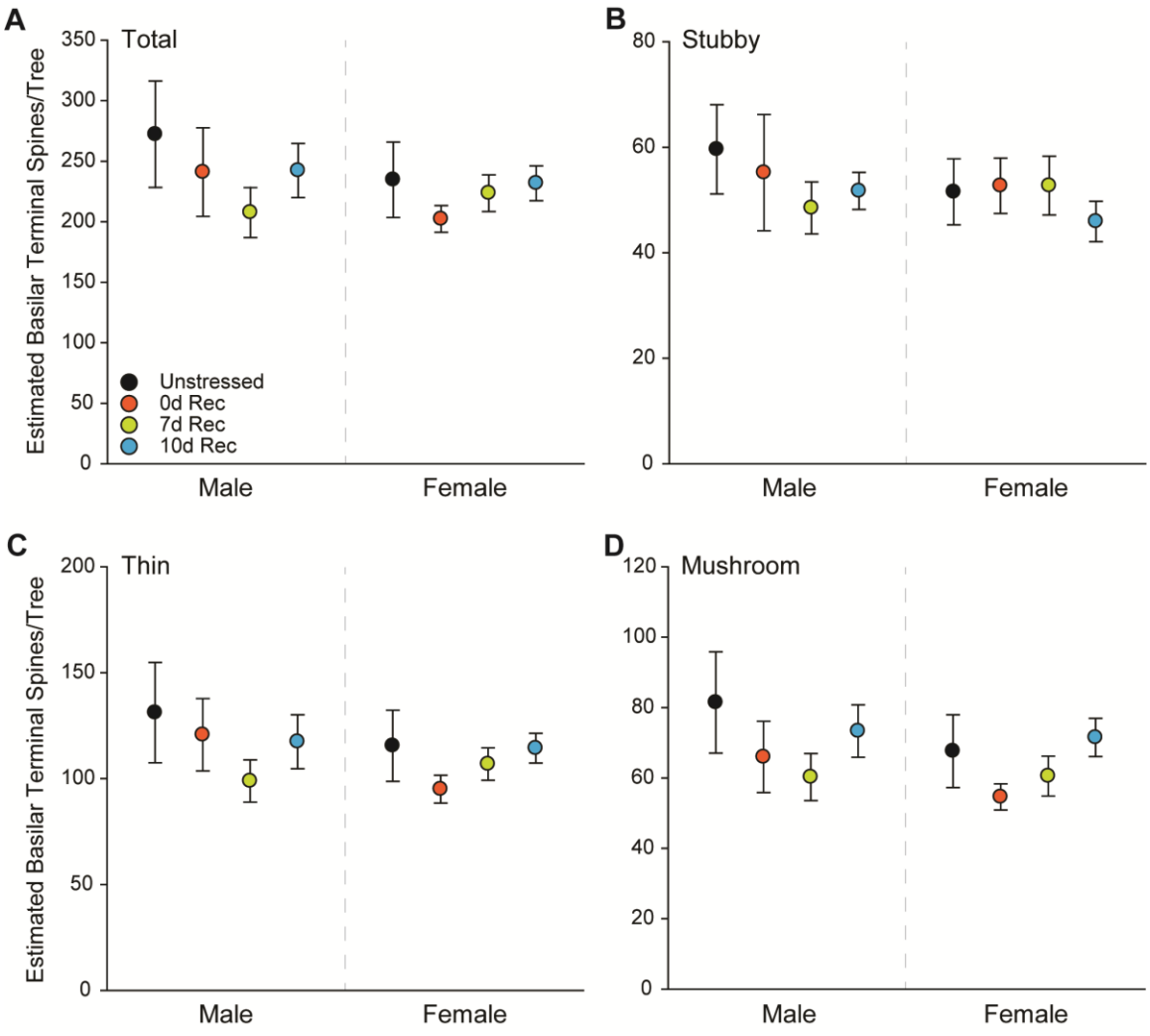


Figure 2.8. Estimated number of (A) total, (B) stubby, (C) thin, and (D) mushroom basilar terminal spines in unstressed 0d, 7d, and 10d Rec male and female rats. Error bars represent SEM.

Discussion

These data indicate that chronically stressed male and female rats have different patterns of dendritic reorganization in prelimbic cortex during the post-stress rest period.

Dynamic Dendritic Reorganization during Stress Recovery in Male Rats

Consistent with previous work (Cook and Wellman, 2004; Radley et al., 2004; Liu and Aghajanian, 2008; Garrett and Wellman, 2009), chronic restraint stress attenuated weight gain and increased relative adrenal weight, suggesting that the manipulation was stressful. Further, relative adrenal weight and body weight gain were normalized by 7 days post-stress. Also consistent with previous studies (e.g., Cook and Wellman, 2004; Garrett and Wellman, 2009), chronic stress induced apical dendritic retraction in the medial prefrontal cortex of male rats. However, apical dendritic length resembled that of unstressed rats by 10 days post-stress. This agrees with previous work demonstrating that dendritic retraction in hippocampal area CA3 resulting from 21 days of restraint stress was reversed by 10 days post-stress (Conrad et al., 1999). Further, our findings expand on Radley and colleagues' (2005) finding that the apical dendritic retraction induced by chronic restraint stress (6 hours/day for 21 days) was fully reversed by 21 days post-stress. Our results indicate that this reversal can happen quite quickly. However, note that in the present study, daily restraint was only 3 hours in duration and occurred for only 10 days. Thus, it is possible that a longer, more intense stressor may result in a different time course of recovery than that found here. In addition, a recent study found that acute stress produced apical dendritic retraction 24 hours and 14 days later, but not at 7 days post-stress (Nava et al., 2015). Therefore, although apical dendritic length resembled that of unstressed rats by 10 days post-stress here, it is possible that the dynamic dendritic remodeling that we have shown continues between 10 and 21 days post-stress, and further alterations in apical dendritic organization could be found at later time points.

Perhaps the most surprising finding from the current study is the apical dendritic outgrowth in male rats 7 days post-stress, followed by a return to lengths similar to those seen in unstressed rats by 10 days post-stress. This initial outgrowth and subsequent pruning is reminiscent of the pattern of dendritic remodeling in injury models following unilateral lesions (reviewed in Macias, 2008). For example, following unilateral motor cortex lesions, pyramidal neurons in layer V contralateral to the lesion showed increased dendritic arborization for the first two weeks post-lesion, followed by partial pruning (Jones and Schallert, 1992). Importantly, changes in dendritic morphology correlated with functional return of the affected limb (Jones and Schallert, 1992, 1994), suggesting that branching patterns, branch distribution, and overall shape of the dendritic arbor can determine the functional properties of neurons. Indeed, this structure-function relationship has been demonstrated in several models (Rall et al., 1992; Mainen and Sejnowski, 1996; Koch and Segev, 2000; Lu et al., 2001; Grudt and Perl, 2002). Thus, although outside the scope of the current study, it is possible that the dendritic reorganization in male rats here has important functional consequences.

One potential functional consequence of the dendritic reorganization shown here is altered HPA axis regulation. Activation of prelimbic cortex has a net inhibitory effect on stress-evoked HPA axis activation via projections to the fusiform and dorsomedial subdivisions of the bed nucleus of the stria terminalis, which in turn send inhibitory projections to the periventricular nucleus of the hypothalamus (PVN; Radley et al., 2009). Interestingly, Ostrander and colleagues (2006) showed that 7 days post-chronic variable stress, males showed HPA axis hypoactivation in response to a novel acute stressor, which was associated with decreased c-fos expression in the PVN . Therefore, the outgrowth of apical dendrites in PL at 7 days post-stress in males could increase the excitability of these cells (Wilber et al., 2011), thereby inhibiting HPA axis activity. This is consistent with the findings described above (Ostrander et al., 2006; Ostrander et al., 2009), as well as the slight reduction in baseline corticosterone seen in the current study. Alternatively, there is evidence that chronic stress-induced dendritic retraction in hippocampus

produces more excitable neurons (Kole et al., 2004). Therefore, increased dendritic length could decrease the excitability of neurons due to a decrease in membrane resistance. Thus, future studies should assess the functional consequences of the dendritic reorganization within PL during the recovery period following chronic stress.

Dendritic Reorganization of Prelimbic Cortex in Female Rats

In contrast to the dynamic dendritic reorganization in PL of male rats, females showed only marginally significant dendritic retraction of apical dendrites in PL. This finding is in contrast to the apical dendritic outgrowth in mPFC of female rats we previously demonstrated (Garrett and Wellman, 2009). Methodological differences between the two studies may account for this discrepancy. First, in the present study, analyses were restricted to PL, whereas the Garrett and Wellman study examined both anterior cingulate cortex and PL. Stress can have opposing effects on various subregions of prefrontal cortex, at least in males (e.g., Liston et al., 2006). Therefore, it is possible that the increase in apical dendritic length found in the previous study could be primarily driven by changes in anterior cingulate, rather than PL. Further, a 10 day chronic restraint paradigm was used here, as opposed to the 7 day stressor used in the previous study. Stressors of different lengths and intensities can have varying effects on dendritic morphology in other brain regions (Vyas et al., 2002; McLaughlin et al., 2007; Maroun et al., 2013; Grillo et al., 2015). Thus, perhaps in females, shorter-term chronic stressors produce dendritic growth, whereas longer-term chronic stressors produce dendritic retraction. Finally, different criteria for selecting neurons were used in this study. Whereas the previous study limited selection to neurons on which all basilar trees were nontruncated and unobstructed, we selected from neurons that had at least one basilar tree that met these criteria, and thus were likely reconstructing neurons from a subpopulation of larger pyramidal cells.

While females in this study did show some degree of apical dendritic retraction, it was small in magnitude compared to that seen in males. Further, the pattern of retraction was different,

with the retraction occurring more proximally in the arbor (see Fig 7). Thus, the effect of chronic stress on prelimbic cortex in males and females is different, with females seeming to be less affected by the deleterious effects of stress on dendritic morphology. This is in agreement with a growing body of literature that suggests that females may be more resilient to stress, in terms of both neuronal morphology and behavior. For example, following 21 days of restraint stress, CA3 pyramidal neurons in males undergo pronounced retraction of apical dendrites, whereas no changes are seen in the apical dendrites of female rats (Galea et al., 1997). Further, following chronic stress, males show behavioral deficits in a variety of spatial and non-spatial memory tasks including object placement (Beck and Luine, 2002), radial arm maze (Luine et al., 1994), Y-maze (Conrad et al., 1996), and object recognition (Beck and Luine, 1999). In contrast, females show either no change (Beck and Luine, 2002), or enhancements in several of these tasks (Bowman et al., 2001; Bowman et al., 2002; McLaughlin et al., 2005). To complicate this, however, in behavioral paradigms in which females initially outperform males (e.g., eyeblink conditioning), stress has the opposite effect. For example, exposure to brief restraint with concomitant tail shock facilitates eyeblink conditioning in males, but impairs conditioning in females (Wood and Shors, 1998). Thus, sex differences in the effects of stress on neuronal morphology and behavior seem to be stressor- and learning paradigm-dependent. Given this, it is possible that males and females are differentially sensitive to restraint stress, and therefore, the sex-specific dendritic reorganization that we observed may be a result of differences in perceived stressor intensity. Furthermore, given that all rats were group-housed and restraint occurred in the home cages alongside restrained cagemates, the sex-dependent stress effects found in the present study could be due to differential social buffering. Both possibilities deserve considerable attention in future studies.

Alterations in Apical and Basilar Spine Density

In addition to apical dendritic retraction immediately following chronic stress, neurons in PL of male rats also exhibited a decrease in the density of stubby spines. Several previous studies have shown decreases in total apical spine density in PL following chronic stress (Radley et al., 2006; Liu and Aghajanian, 2008; Hains et al., 2009), and at least one prior study has suggested that this loss of dendritic spines is driven primarily by a decrease in the density of larger spines (Radley et al., 2008). As larger spines are considered more stable (Rochefort and Konnerth, 2012), this suggests that chronic stress impedes spine stabilization. As we classified spines by morphology as opposed to size, it is difficult to draw direct comparisons between our results and these previous findings. Stubby spines are considered to be the least mature spine type on cortical pyramidal neurons, whereas thin and mushroom spines are thought to be more stable (Rochefort and Konnerth, 2012). Thus, a decrease in stubby spines without a concurrent increase in thin or mushroom spine density suggests a lack of spine stabilization following exposure to chronic stress, which is consistent with the notion that stress may decrease spine lability. This lack of concurrent increase in thin and mushroom spine densities combined with decreased dendritic length resulted in a net loss of spines, which likely has important implications for the function of these neurons immediately post-stress. Consistent with this notion, we previously found that stubby spine density in the IL region of mPFC correlates quite strongly with stress-induced extinction deficits (Moench et al., 2016).

To our knowledge, this is the first study examining changes in spine density of PL neurons in females following chronic stress. Immediately post-stress, decreases in the number but not density of thin and mushroom spines were seen in females, a dissociation likely due to subtle remodeling of apical dendrites. In contrast to the decreased density and estimated total number of stubby spines on apical terminal dendrites observed in male rats immediately following chronic stress, stressed female rats showed no change in density immediately following stress. Following

a 7 day recovery period, both density and estimated total number of stubby spines had increased. This suggests that in the days following the cessation of stress, females, in contrast to males, may have an increase in spine lability in PL. As spines play an important role in the information processing of neurons, these lasting changes in spine density in the days following stress likely have important consequences for the overall functioning of PL, even several days after the cessation of stress, and again demonstrates sex differences in the lasting effects of stress in this region.

In agreement with previous findings from our lab and others, chronic stress did not alter the morphology of basilar dendritic arbors in PL of either males or females (Cook and Wellman, 2004; Radley et al., 2006; Garrett and Wellman, 2009). These previous findings have led to the general conclusion that, unlike apical dendrites, basilar dendrites in mPFC may be insensitive to the effects of stress. In contrast to this notion, however, here we found alteration in basilar spine density as a result of chronic stress. Specifically, neurons in PL of male rats had a decrease in overall spine density on basilar dendrites at 7 days post-stress, suggesting that changes in basilar spines may occur over a different timescale than that of apical spines. Further, both males and females exhibited a stress-induced decrease specifically in mushroom spines immediately after stress, which neared significance following 7 days of recovery. Thus, basilar dendrites may not be as impervious to stress as once thought. Further, there is some evidence that changes in basilar dendrite morphology and spine density may be hemisphere-specific and may vary by the time of day during which stress is administered (Perez-Cruz et al., 2009), indicating stress-induced changes in basilar dendrites may be more nuanced than those changes observed in apical dendrites. Indeed, this is consistent with the lack of significant alteration in estimates of total basilar spines, which suggests subtle changes in basilar dendritic length that countered the alterations in spine densities may have occurred. As pyramidal neurons segregate their inputs (Spratling, 2002), these changes in apical basilar dendritic spines suggest differential processing of input by each tree type in response to chronic stress.

Sex Differences in Stress-induced Alterations in Basal HPA Axis Activity

HPA axis dysregulation is a common feature of several stress-related psychological disorders. For example, hypercortisolism has long been implicated in depression and bipolar disorder (Carroll, 1982; Rush et al., 1996; Rybakowski and Twardowska, 1999; Maripuu et al., 2014), whereas hypocortisolism is more often associated with PTSD (Thaller et al., 1999; Yehuda et al., 2000; Kirschbaum et al., 2002; Rohleder et al., 2004). It has yet to be determined whether HPA dysregulation presents a risk factor for pathology or if it is part of the symptomatology of a given disorder. Given the numerous etiologies of stress-related disorders, both cases are likely. Here, we found that, whereas previously-stressed males tended to show attenuated baseline levels of serum corticosterone at 0 and 7 days post-stress, previously-stressed females had remarkably higher levels of corticosterone, even at 10 days post-stress. The trend toward corticosterone suppression in males agrees with previous work from Ostrander and colleagues (2006) who demonstrated that one week following chronic variable stress, male rats had attenuated adrenocorticotropic hormone and corticosterone levels following a heterotypic acute stressor, although they did not find significant basal differences in corticosterone at this time point. This difference could reflect the use of a different stress paradigm in the current study, but nonetheless suggests a possible trend in HPA axis hypoactivity in males following chronic stress.

Several mechanisms could underlie basal HPA axis hypoactivity in males. One possibility involves corticotropin releasing hormone (CRH) production and its relationship with HPA axis responsivity. During chronic stress, CRH production in the PVN increases. Sustained hypersecretion of CRH results in CRH receptor downregulation in the pituitary (Hauger et al., 1988; Aguilera et al., 2001). In the recovery period following the cessation of stress when CRH levels return to normal, receptor expression is still relatively low. Thus, attenuated CRH secretion, in combination with fewer pituitary CRH receptors, will lead to less adrenocorticotropic hormone (ACTH) production, and therefore, less corticosterone secretion. In support of this hypothesis,

mice lacking CRH receptors lack the ability to mount a proper HPA axis response to stress (Smith et al., 1998). Therefore, the corticosterone suppression found here in males could represent a period of time in the recovery process prior to the normalization of pituitary CRH receptors.

Not only did females show starkly different corticosterone levels throughout the recovery period compared to males, there was also a high degree of variability in females that was not observed in males (see Fig. 2.3). Importantly, this variability was not due to the hormonal status of the females. One possible explanation for this variability could be the pulsatile nature of corticosterone release in females. Compared to males, females show larger and more frequent peaks of corticosterone throughout a 24 h period (Seale et al., 2004). As such, it is possible that some blood samples may have been collected at or near a peak of corticosterone secretion, while others were collected when corticosterone levels were relatively low. This seems unlikely, as unstressed females did not show the same variability as females in all three of the stressed groups. Instead, chronic stress resulted in HPA axis dysregulation that altered basal corticosterone levels in a subset of females. This suggests that, even within rodents, females may have a high degree of individual variation in recovery from stress. Understanding this sex-dependent variation in stress responsivity could elucidate risk factors that may have profound effects on HPA axis adaptability, and thus responsivity to future stressors, and risk for stress-induced psychopathology.

Conclusions

These data demonstrate sex differences in dendritic reorganization in prelimbic cortex during the post-chronic stress rest period, whereby male rats have a dynamic pattern of dendritic changes in the days following chronic stress. In contrast, female rats show a lesser degree of dendritic changes. This finding raises the interesting possibility that prelimbic cortex of males and females may be functioning differently during this post-stress time.

Chapter 3:

Chronic stress produces enduring sex- and region-specific alterations in novel stress-induced c-Fos expression.

Moench, K.M., Breach, M.R., Wellman, C.L. (2019). Neurobiology of Stress, 10, 100147.

Initial investigations of the post-stress rest period in rodents suggested that stress-induced changes in the brain are completely reversible. For instance, chronic stress-induced dendritic atrophy of pyramidal neurons in CA3 and medial prefrontal cortex (mPFC) is ameliorated following a rest period (Conrad et al., 1999; Radley et al., 2005; Moench and Wellman, 2017), as are deficits in spatial working memory (Sousa et al., 2000). Findings from more recent studies, however, suggest that changes in the brain during the post-stress time period do not constitute a simple return to pre-stress conditions, but instead result in a distinct functional state.

In the case of mPFC, novel stress-induced c-*fos* mRNA is blunted in male rats 16 hours following the cessation of 14 days of chronic variable stress (Ostrander et al., 2009). This blunted response was also observed following a 30 day post-stress rest period, suggesting there are relatively long-lasting changes in the post-stress function of mPFC. Given that dendritic remodeling in mPFC during the post-stress rest period is sex-specific (Chapter 2; Moench and Wellman, 2017), it is likely that the response of mPFC to a novel stressor during this time also differs between males and females. To test this hypothesis, I exposed chronically stressed male and female rats to a novel acute stressor either 1 or 7 days following the cessation of chronic stress. Using c-Fos expression, I assessed changes in acute stress-induced neuronal activation of several corticolimbic regions that are important in cognitive and emotional processing, as well as regulation of stress physiology: the prelimbic (PL) and infralimbic (IL) subregions of mPFC; orbitofrontal cortex (OFC); basolateral amygdala (BLA); dorsal hippocampus subfields CA1, CA3 and dentate gyrus (DG); and the paraventricular nucleus of the hypothalamus (PVN).

Materials and Methods

Subjects and Stressors

Male and female Sprague Dawley rats (approximately 10 weeks of age at start; Envigo, Indianapolis, IN) were group-housed (3/cage) in standard laboratory cages (48 cm × 20 cm × 26 cm), with ambient temperature 23-25 °C, free access to food and water, and a 12:12 light/dark cycle (lights on at 0800 h). All procedures were conducted between 8:00 am and 6:00 pm, were in accordance with NIH Guidelines, and were approved by Indiana University's IACUC.

Rats were assigned to one of four stress conditions (see Fig. 3.1 for experimental design and timeline): No Stress (n = 6/sex); Elevated platform stress only (EPS Only; n = 9/sex); chronic stress followed by EPS 1 day later (CRS-EPS; n = 12 male; 9 female); or chronic stress followed by EPS 7 days later (CRS-Rest-EPS; n = 9 male; 8 female). Chronic stress consisted of daily restraint (3 h/day, 10 d). Rats were weighed daily throughout the stress procedure. Immediately after weighing, unstressed rats were returned to their home cages and left undisturbed in a separate room. Chronically stressed rats were placed in semi-cylindrical restrainers (male, 16 cm length × 6.5 cm width × 5 cm height; female, 15 cm length × 6 cm width × 4.5 cm height, modified so the tail piece locks into place; Braintree Scientific, Braintree, MA) in their home cages, with the time of restraint unpredictably varied over the light cycle. This manipulation produces significant increases in plasma corticosterone levels (Cook and Wellman, 2004). EPS consisted of placing each rat individually on a small platform (12 cm × 12 cm) elevated 90 cm off the ground for 30 min in a brightly lit room as previously described (Xu et al., 1997; Maroun and Richter-Levin, 2003; Maroun et al., 2013).

On the day of perfusion, vaginal lavages were performed and exfoliate cytology was examined immediately under light microscopy. Estrous phase was determined based on the morphology of cells present (Garrett and Wellman, 2009). Due to the small number of rats in proestrus and estrus (n = 1, No Stress; 2, EPS Only; 3, CRS-EPS; 3, CRS-Rest-EPS), we did not analyze our data relative to estrous phase.

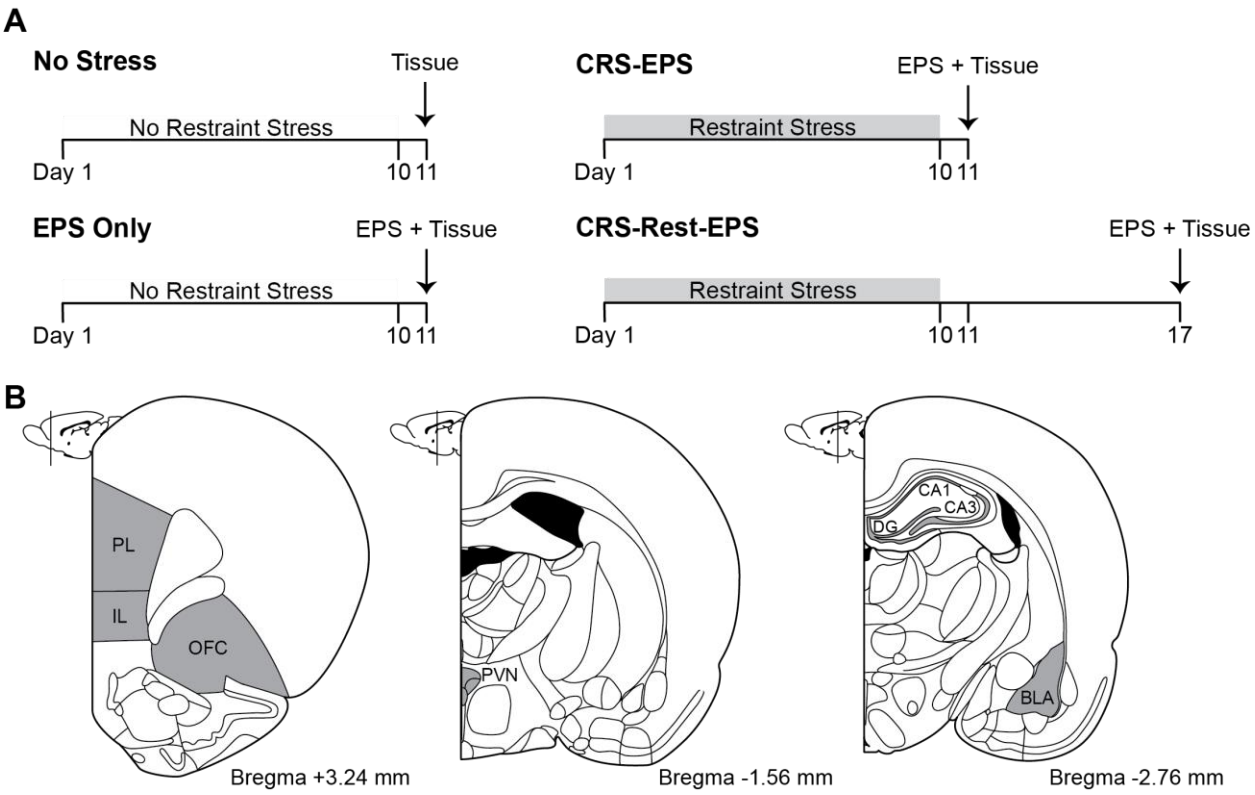


Figure 3.1. (A) Experimental design and timeline. Male and female rats were either left undisturbed or were exposed to CRS for 10 days. Rats either remained unstressed (No Stress) or were exposed to EPS (EPS Only). CRS rats were exposed to EPS either on the day following CRS (CRS-EPS) or 7 days following the cessation of CRS (CRS-Rest-EPS). (B) Schematic diagrams identifying regions examined. c-Fos expression was analyzed in PL, IL, OFC, PVN, CA1, CA3, DG, and BLA. Adapted from Paxinos and Watson (2007).

Immunohistochemistry

Approximately 60 min after the cessation of EPS, rats were deeply anesthetized with urethane and transcardially perfused with cold 0.1 M phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Brains were removed and hemisected. One hemisphere per animal, counterbalanced across animals, was post-fixed in 4% PFA for 24 h. Hemispheres were cryoprotected in 30% sucrose and sectioned frozen at 44 µm on a sliding microtome and collected into 0.1 M PBS. For each hemisphere, 5 to 6 sections (approximately 264 µm apart) were collected through the rostral-caudal extent of mPFC, 3 to 4 sections (approximately 176 µm apart) were collected through the PVN, and 3 to 4 sections (approximately 396 µm apart) through DHC and BLA.

Free-floating sections were stained for c-Fos using a modification of the protocol described by Lenz et al. (2010). Sections were incubated in blocking solution (0.1% Triton X-100, 5% NGS, and 5% bovine serum albumin in PBS) and 1% hydrogen peroxide for 30 min to block nonspecific binding and endogenous peroxidases. Sections were then incubated for 48 h at 4 °C in blocking solution and a rabbit polyclonal antibody for c-Fos (1:2000, Santa Cruz Biotech, sc-253). After rinsing, sections were incubated for 1 h in blocking solution and biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, USA). Sections were rinsed and incubated for 1 h in PBS with 0.1% Triton X-100 and ABC complex (Vector Laboratories). Staining was visualized using a nickel-intensified DAB reaction. After rinsing, sections were mounted, dehydrated, cleared, and coverslipped. Control sections incubated without the primary antibody demonstrated no staining.

To investigate the potential neuroendocrine ramifications of altered c-Fos expression in the PVN, we used a procedure similar to that of Smith et al. (2016) to perform double-label immunohistochemistry for c-Fos and corticotropin-releasing hormone (CRH) in a separate set of EPS Only, CRS-EPS, and CRS-Rest-EPS rats (n = 3/group/sex). Brains were prepared as described above. For immunohistochemistry, free-floating sections were incubated in blocking

solution (0.1% Tween 20 and 5% NGS in 0.1 M PBS), followed by incubation 48 h at 4 °C in blocking solution with a polyclonal anti-CRH antibody raised in guinea pig (Peninsula Labs, 1:10,000). After rinsing, sections were incubated 1 h in blocking solution and biotinylated goat anti-guinea pig (1:200, Vector Laboratories). Sections were rinsed and incubated 1 h in PBS with 0.1% Triton X-100 and ABC complex (Vector Laboratories). CRH was visualized using a DAB reaction. Staining for c-Fos then proceeded as described above. Control section incubated without primary antibodies demonstrated no staining.

Stereology

We examined the density of c-Fos+ cells in PL, IL, and OFC; the pyramidal cell layer of CA1 and CA3 and the granule cell layer of DG; BLA; and PVN. In addition, we examined the density of c-Fos+, CRH+, and c-Fos+/CRH+ cells in the PVN. Sampling occurred throughout the anterior-posterior extent of each region and was completed at a final magnification of 1800× using the optical fractionator method and Stereo Investigator (MBF Biosciences Inc., Williston, VT). The counting frame for all regions was 50 µm × 50 µm. Sampling grid size for each region was as follows: PL, 150 µm × 300 µm; IL, 200 µm × 200 µm; OFC, 300 µm × 300 µm; CA1, 150 µm × 150 µm; CA3, 150 µm × 150 µm; DG, 150 µm × 150 µm; BLA, 150 µm × 250 µm; PVN, 100 µm × 100 µm. Guard zones were set with a centered-probe thickness of 15 µm for each region. Counts were performed with the experimenter blind to sex and stress condition.

Statistical Analyses

Chronic stressors such as immobilization and restraint attenuate normal weight gain (Marti et al., 1994; Cook and Wellman, 2004). To verify our chronic stress manipulation, weight change (start weight – weight on the final day of restraint) was compared between stress conditions using a two-way ANOVA (sex × stress), followed by Fisher's protected LSD *post hoc* comparisons.

For all brain regions, the estimated density of c-Fos+ cells was calculated and analyzed using two-way ANOVAs (sex × stress). Significant effects were followed by Fisher's protected LSD *post hoc* comparisons. To assess the co-localization of c-Fos in CRH+ cells in the PVN, we calculated the percentages of cells labeled only for c-Fos (c-Fos+ cells), cells labeled only for CRH (CRH+ cells) and cells double-labeled for c-Fos and CRH (c-Fos+/CRH+ cells). These data were analyzed using three-way repeated measures ANOVAs (immunolabeling × stress × sex, immunolabeling as the repeated measure), followed by Fisher's protected LSD *post hoc* comparisons. Additionally, Spearman correlations were used to examine potential relationships of basal and acute stress-induced c-Fos expression between all brain regions examined.

Results

Body Weight Analysis

Weight comparisons included rats used for both single- and double-label immunohistochemistry. Male rats gained significantly more weight than female rats (Fig. 3.2; main effect of sex, $F_{(1, 78)} = 23.97$, $p < 0.001$) and stress altered weight change in both males and females (main effect of stress, $F_{(3, 78)} = 53.19$, $p < 0.001$), although this effect was more pronounced in males (sex × stress interaction, $F_{(3, 78)} = 6.77$, $p < 0.001$). In males, weight gain did not differ between No Stress and EPS Only rats. In contrast, male rats exposed to chronic stress gained significantly less weight than No Stress male rats (CRS-EPS, $p < 0.001$; CRS-Rest-EPS, $p < 0.001$). Similarly, in female rats, weight change did not differ between No Stress and EPS Only rats, whereas in chronically stressed females, weight change was significantly reduced relative to No Stress female rats (CRS-EPS, $p < 0.001$; CRS-Rest-EPS, $p = 0.004$).

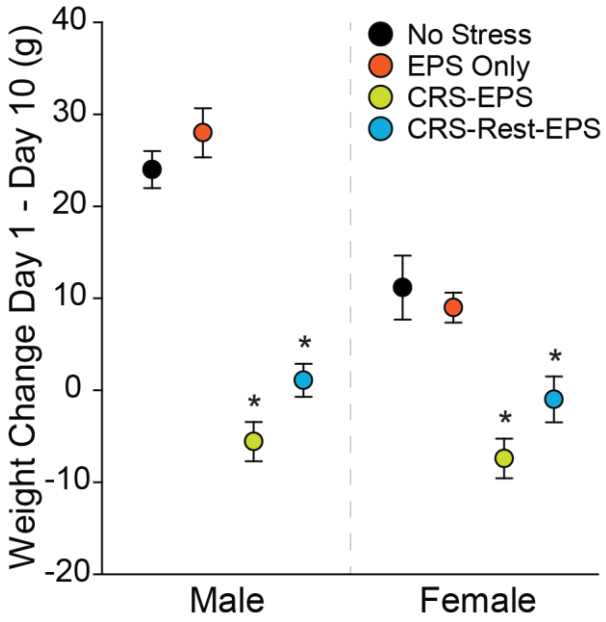


Figure 3.2. Chronic stress attenuates weight gain in both male and female rats. Error bars represent SEM. * $p < 0.05$ compared to No Stress rats of same sex.

c-Fos Expression: Prefrontal Cortex

For mPFC, animals were excluded from analyses when hemisection resulted in tissue damage to the region of interest. Thus, final n's for PL were as follows: male No Stress, n = 5; EPS Only, n = 7; CRS-EPS, n = 11; CRS-Rest-EPS, n = 9; and female No Stress, n = 5; EPS Only, n = 8; CRS-EPS, n = 8, CRS-Rest-EPS, n = 6. Final n's for IL were as follows: male No Stress, n = 5; EPS Only, n = 7; CRS-EPS, n = 11; CRS-Rest-EPS, n = 7; and female No Stress, n = 5; EPS Only, n = 8; CRS-EPS, n = 8, CRS-Rest-EPS, n = 6.

In PL (Fig. 3.3 B), females had greater overall c-Fos expression than males (main effect of sex, $F_{(1, 51)} = 6.26$, $p = 0.02$). Stress significantly altered the density of c-Fos+ cells (main effect of stress, $F_{(3, 51)} = 21.61$, $p < 0.001$), although the interaction between sex and stress was not significant ($F_{(3, 51)} = 0.83$, n.s.). Follow up comparisons revealed that the density of c-Fos+ cells did not differ between No Stress male and female rats. In males, EPS significantly increased c-Fos expression compared to No Stress males (p's < 0.001), although this increase was attenuated in male rats previously exposed to CRS (EPS Only v CRS-EPS, $p = 0.001$; EPS Only v CRS-Rest-EPS, $p = 0.02$). This effect was not dependent on a rest period, as the CRS groups did not

differ from each other. EPS also resulted in increased c-Fos expression in female rats (p's ≤ 0.001), but this was not altered by previous chronic stress exposure regardless of rest period.

In IL (Fig. 3.3 C), the density of c-Fos+ cells was significantly higher in females than males (main effect of sex, $F_{(1, 49)} = 8.25$, $p = 0.01$), and was altered by stress (main effect of stress, $F_{(3, 49)} = 13.13$, $p < 0.001$), although the interaction of sex and stress was not significant ($F_{(3, 49)} = 2.13$, n.s.). Follow up comparisons indicated a non-significant trend towards increased c-Fos expression in No Stress females compared to males ($p = 0.06$). In males, EPS increased c-Fos expression in all stress groups (p's < 0.001). This effect was blunted in CRS males not given a rest period (EPS Only v CRS-EPS, $p = 0.01$), but not in those with a rest period. Further, CRS-Rest-EPS had greater c-Fos expression that CRS-EPS males ($p = 0.03$). EPS also increased the density of c-Fos+ cells in all female stress conditions (p's ≤ 0.01), although this was not altered by previous chronic stress exposure regardless of rest period.

In OFC (Fig. 3.3 D), females tended to have greater overall c-Fos expression than males (main effect of stress, $F_{(1, 60)} = 2.88$, $p = 0.09$). Further, stress altered c-Fos expression (main effect of stress, $F_{(3, 60)} = 26.72$, $p < 0.001$), and this effect differed between males and females (sex × stress interaction, $F_{(3, 60)} = 3.68$, $p = 0.02$). Follow up comparisons indicated that c-Fos expression did not differ between No Stress male and females. In males, EPS significantly increased c-Fos expression across all stress conditions (p's < 0.001). Although CRS-EPS males did not differ from EPS Only males, CRS-Rest-EPS males tended to have enhanced c-Fos expression compared to EPS Only males ($p = 0.09$). Further, CRS-Rest-EPS males had a significantly greater density of c-Fos+ cells compared to CRS-EPS ($p = 0.001$). In females, EPS significantly increased c-Fos expression in all stress conditions (p's < 0.001). This effect was blunted in all chronically stressed females regardless of rest period (EPS Only v CRS-EPS, $p = 0.002$; EPS Only v CRS-Rest-EPS, $p = 0.005$).

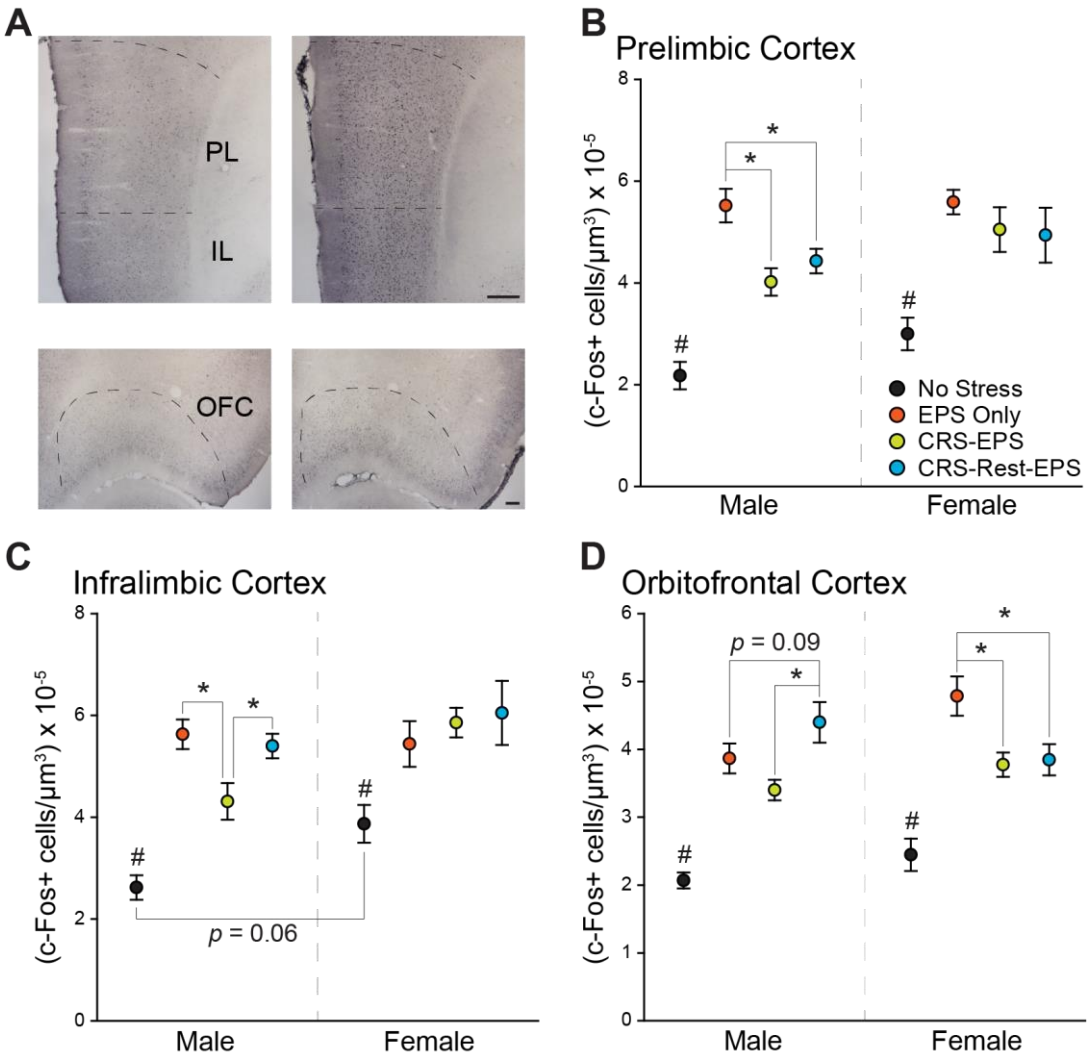


Figure 3.3. (A) Representative photomicrographs of c-Fos immunohistochemistry in PL and IL (*Top*) and OFC (*Bottom*) of No Stress (*Left*) and EPS Only (*Right*) male rats. Scale bar = 250 μm. (B) In both male and female rats, EPS increases c-Fos expression in PL. This increase is reduced in both CRS male groups, but is unaltered in CRS female groups. (C) Female rats tended to have greater basal c-Fos expression in IL compared to male rats. In both male and female rats, EPS increases c-Fos expression in IL. This increase is reduced in CRS-EPS males, but is unaltered in CRS-Rest-EPS males. In female rats, EPS-induced c-Fos expression is unaltered in both CRS groups. (D) In both male and female rats, EPS increases c-Fos expression in OFC. In males, this increase is slightly enhanced in CRS males given a rest period. In female rats, EPS-induced c-Fos expression is reduced in both CRS groups. Error bars represent SEM. * $p < 0.05$; # $p < 0.05$ compared to all other stress conditions of same sex.

c-Fos Expression: Hippocampus

In CA1 (Fig. 3.4 B), female rats had greater c-Fos expression than males (main effect of sex, $F_{(1, 60)} = 5.70$, $p = 0.02$). Stress altered the density of c-Fos+ cells (main effect of stress, $F_{(3, 60)} = 31.88$, $p < 0.001$), and this effect differed between males and females (sex × stress interaction, $F_{(3, 60)} = 3.35$, $p = 0.03$). In males, EPS increased c-Fos expression across all stress conditions (p's ≤ 0.004). Prior chronic stress, regardless of rest period, blunted this increase (EPS Only v CRS-EPS, $p = 0.02$; EPS Only v CRS-Rest-EPS, $p = 0.001$). EPS also increased c-Fos expression across all stress conditions in females (p's < 0.001), but this effect was not altered by chronic stress at either timepoint.

In CA3 (Fig. 3.4 C), the density of c-Fos+ cells did not differ between males and females (main effect of sex, $F_{(1, 60)} = 0.15$, n.s.). Stress altered c-Fos expression (main effect of stress, $F_{(3, 60)} = 12.72$, $p < 0.001$), although this effect did not differ between males and females (sex × stress interaction, $F_{(3, 60)} = 0.21$, n.s.). In both males and females, EPS increase c-Fos expression across all stress conditions (p's < 0.01), although there were no differences between stress conditions.

In DG (Fig. 3.4 D), the density of c-Fos+ cells did not differ between males and females (main effect of sex, $F_{(1, 60)} = 0.14$, n.s.). Stress significantly altered c-Fos expression (main effect of stress, $F_{(3, 60)} = 27.26$, $p < 0.001$), and this effect differed between males and females (sex × stress interaction, $F_{(3, 60)} = 5.04$, $p = 0.003$). Follow up comparisons indicated that in males, EPS increased c-Fos expression across all stress conditions (p's < 0.01). This increase was blunted in all males exposed to CRS (EPS Only v CRS-EPS, $p < 0.001$; EPS Only v CRS-Rest-EPS, $p = 0.04$), although this was more pronounced in CRS-EPS males (CRS-EPS v CRS-Rest-EPS, $p = 0.05$). In females, EPS also resulted in increased c-Fos expression across all stress conditions (p's < 0.001). This was largely unchanged by chronic stress, although a non-significant trend towards an increase in EPS-induced c-Fos expression was observed (EPS Only v CRS-Rest-EPS, $p = 0.09$). CRS-EPS and CRS-Rest-EPS females did not differ from each other.

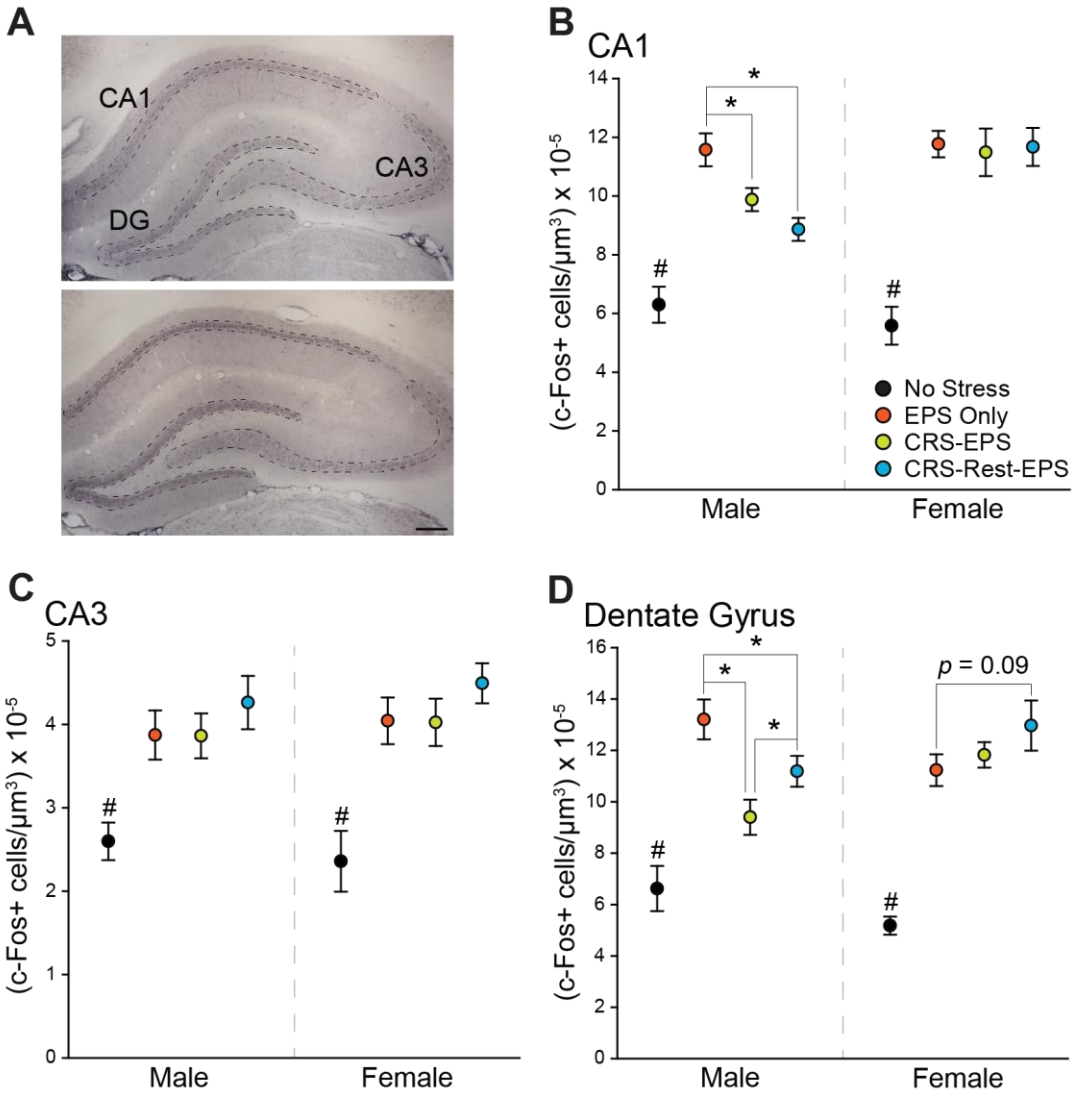


Figure 3.4. (A) Representative photomicrographs of c-Fos immunohistochemistry in CA1, CA3, and DG of No Stress (*Top*) and EPS Only (*Bottom*) male rats. Scale bar = 250 μm. (B) In both males and females, EPS increases c-Fos expression in CA1. This increase is reduced in both CRS male groups, but is unaltered in CRS female groups. (C) In both male and female rats, EPS increases c-Fos expression in CA3. This increase in unaltered by CRS in both males and females. (D) In both males and females, EPS increases c-Fos expression in DG. This increase is reduced in both CRS male groups, but is unaltered in CRS female groups. * $p < 0.05$. $^{\#}$ $p < 0.05$ compared to all other stress conditions of same sex. Error bars represent SEM.

c-Fos Expression: Basolateral Amygdala

In BLA (Fig. 3.5), female rats had greater c-Fos expression compared to males (main effect of sex, $F_{(1, 60)} = 5.49$, $p = 0.02$). Stress altered the density of c-Fos+ cells (main effect of stress, $F_{(3, 60)} = 31.88$, $p < 0.001$), although this effect did not differ between males and females (sex × stress interaction, $F_{(3, 60)} = 1.32$, n.s.). In males, EPS increased c-Fos expression across all stress conditions (p's ≤ 0.004). Prior chronic stress without a rest period tended to blunt this response (EPS Only v CRS-EPS, $p = 0.08$). Chronically stressed males given a rest period did not differ from EPS Only males, although they tended to have great c-Fos expression then those without a rest period ($p = 0.07$). EPS also increased c-Fos expression across all stress conditions in females (p's < 0.001). This effect was not altered CRS-EPS females. In contrast, CRS-Rest-EPS females had enhanced EPS-induced c-Fos expression (EPS Only v CRS-Rest-EPS, $p = 0.05$), but this increase did not reach significance compared to CRS-EPS rats.

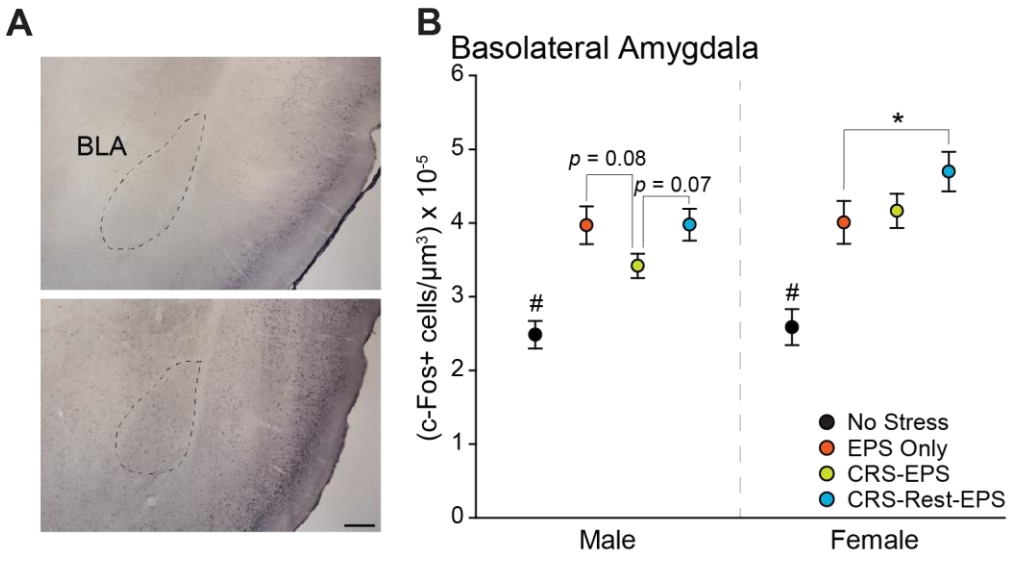


Figure 3.5. (A) Representative photomicrographs of c-Fos immunohistochemistry in BLA of No Stress (*Top*) and EPS Only (*Bottom*) male rats. Scale bar = 250 μm. (B) In male rats, acute stress increases c-Fos expression in BLA. In CRS-EPS males this increase is slightly reduced. In CRS-Rest-EPS females the acute stress-induced increase is enhanced. * $p < 0.05$. $^{\#}$ $p < 0.05$ compared to all other stress conditions of same sex. Error bars represent SEM.

c-Fos Expression: Paraventricular Nucleus

As in mPFC, a small number of animals were excluded from analyses due to tissue damage resulting from hemisection. Thus, final n's for PVN were as follows: male No Stress, n = 5; EPS Only, n = 9; CRS-EPS, n = 9; CRS-Rest-EPS, n = 8; and female No Stress, n = 6; EPS Only, n = 9; CRS-EPS, n = 9, CRS-Rest-EPS, n = 7. The density of c-Fos+ cells in the PVN (Fig. 3.5 6) did not differ between males and females (main effect of sex, $F_{(1, 54)} = 1.10$, n.s.). Stress significantly altered c-Fos expression (main effect of stress, $F_{(3, 54)} = 53.79$, $p < 0.001$), and this effect differed between males and females (sex × stress interaction, $F_{(3, 54)} = 5.21$, $p = 0.003$). Follow up comparisons indicated that in males, EPS increased c-Fos expression across all stress conditions (p's < 0.001). This increase was blunted CRS-EPS males (EPS Only v CRS-EPS, $p < 0.001$), with a non-significant trend in the same direction in CRS-Rest-EPS males (EPS Only v CRS-Rest-EPS, $p = 0.08$), resulting in a significant difference between chronically stressed males without and with a rest period ($p = 0.04$). In females, EPS also resulted in increased c-Fos expression across all stress conditions (p's < 0.001). This effect was not altered CRS-EPS females. In contrast, CRS-Rest-EPS females had enhanced EPS-induced c-Fos expression compared to both EPS Only ($p = 0.002$) and CRS-EPS ($p = 0.002$) females.

c-Fos/CRH Co-expression Analysis

In the PVN, single- versus double-labeled cells were readily discriminable by focusing through the tissue. Three-way repeated measures ANOVA revealed a main effect of immunolabeling in the PVN (Fig. 3.6 C; $F_{(2, 24)} = 483.47$, $p < 0.0001$), but not sex ($F_{(1, 12)} = 0.50$, n.s.) or stress ($F_{(2, 12)} = 3.50$, n.s.). There were no significant interactions (Fs ≤ 3.50, all n.s.). Follow-up pairwise comparisons revealed that there was a higher percentage of CRH+ (62.83 ± 1.41) cells compared to either CRH+/c-Fos+ (33.85 ± 1.17; $p < 0.001$) or c-Fos+ (3.32 ± 0.55; $p < 0.001$) cells. In addition, CRH+/c-Fos+ cells were more significantly more numerous than c-Fos+ cells ($p < 0.001$).

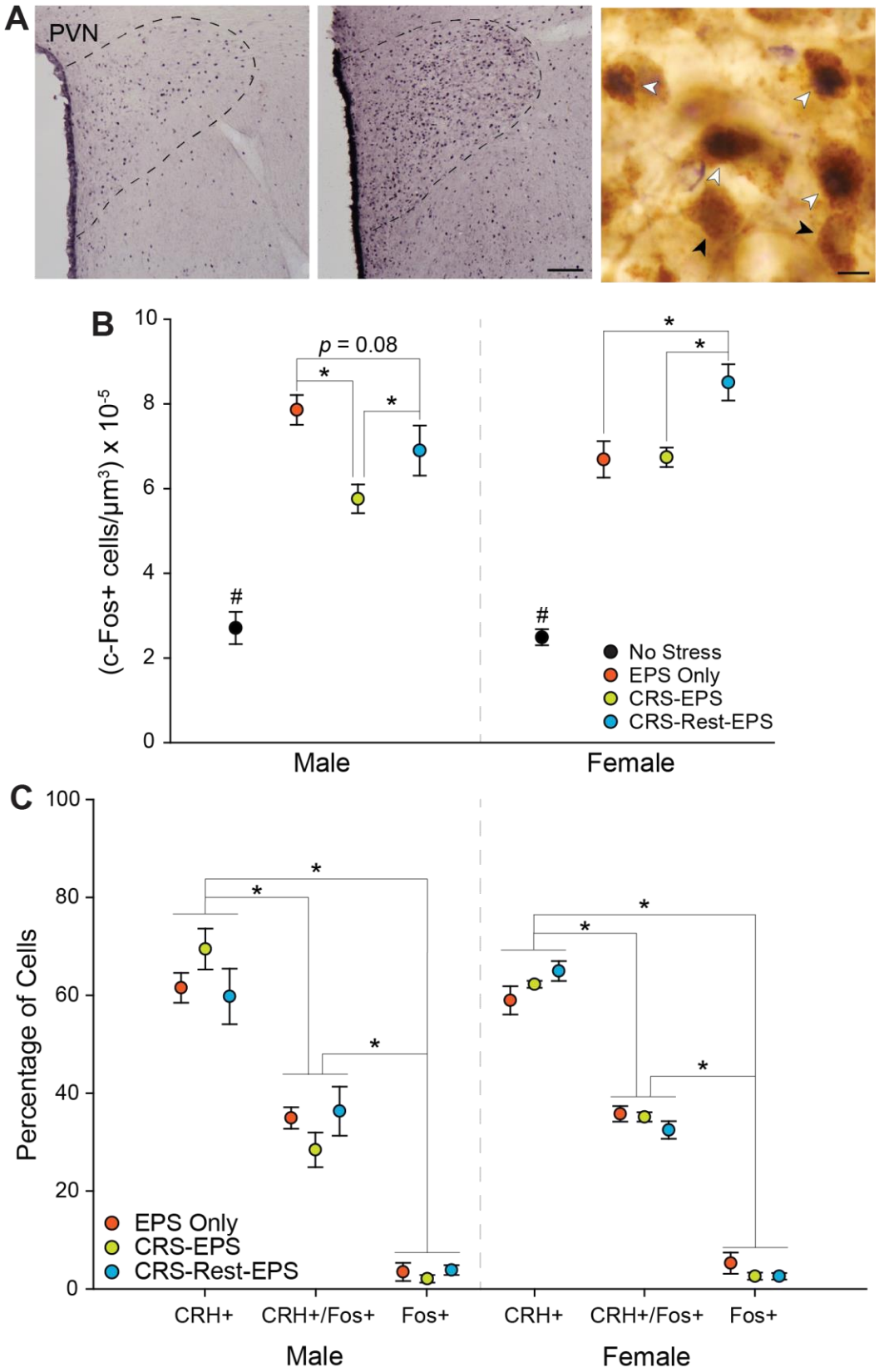

A
PVN
B
(c-Fos+ cells/μm³) x 10⁻⁵
p = 0.08
No Stress
EPS Only
CRS-EPS
CRS-Rest-EPS
Male
Female
C
Percentage of Cells
EPS Only
CRS-EPS
CRS-Rest-EPS
CRH+
CRH+/Fos+
Fos+
Male
Female

Figure 3.6. (A) Representative photomicrographs of c-Fos immunohistochemistry in the PVN of No Stress (*left*) and EPS Only (*middle*) male rats. Scale bar = 100 µm. (*Right)* Photomicrograph of c-Fos (black) and CRH (brown) labeled cells within the PVN. Black arrowheads indicate single-label CRH immunopositive cells. White arrowheads indicate double-labeled cells. Scale bar = 10 µm. (B) Chronic stress effects on EPS-induced c-Fos expression in the PVN in male and female rats. In both male and female rats, EPS increases c-Fos expression in the PVN. This increase is significantly reduced in CRS-EPS male rats, and to a lesser extent in CRS-Rest-EPS males. In females, the acute stress-induced increase is enhanced in CRS-Rest-EPS rats. (C) In male and female rats, the majority of c-Fos immunopositive cells are co-localized with CRH across all stress conditions. * $p < 0.05$. $^{\#}$ $p < 0.05$ compared to all other stress conditions of same sex. Error bars represent SEM.

Inter-region Correlations

The relationships among c-Fos expression across brain regions were examined using correlational analyses (Fig. 3.7). In No Stress male rats, strong and significant positive correlations between c-Fos expression in PL and IL ($r_{(5)}$= 0.98, $p < 0.01$), CA1 and CA3 ($r_{(6)}$ = 0.83, p = 0.04), and CA1 and DG ($r_{(6)}$ = 0.82, p = 0.04) were observed. A negative correlation between OFC and PVN ($r_{(5)}$ = -0.80, p = 0.10) and a positive correlation between BLA and DG ($r_{(6)}$ = 0.78, p = 0.07) approached significance. In No Stress females, c-Fos expression in PL and OFC ($r_{(5)}$ = 0.90, p = 0.04), CA3 and DG ($r_{(5)}$ = 0.87, p = 0.02), and CA3 and BLA ($r_{(5)}$ = 0.89, p = 0.02) were strongly positively correlated, whereas expression in IL and DG ($r_{(5)}$ = -0.90, p = 0.04) and IL and BLA ($r_{(5)}$ = -0.90, p = 0.04) were strongly negatively correlated. Positive correlations between c-Fos expression in CA1 and DG ($r_{(6)}$ = 0.75, p = 0.08) and CA1 and BLA ($r_{(6)}$ = 0.77, p = 0.07) approached significance. Thus, in unstressed male and female rats, the pattern of correlated activity across the corticolimbic regions examined varies in strength and direction.

In EPS only male rats, only one correlation reached significance: c-Fos expression in CA1 and PVN was negatively correlated ($r_{(9)}$ = -0.80, p = 0.01). A positive correlation between PL and IL ($r_{(7)}$ = 0.67, p = 0.10) approached significance, as did a negative correlation between IL and

BLA ($r_{(7)}$ = -0.80, p = 0.07). In EPS Only female rats, c-Fos expression in IL and DG ($r_{(8)}$ = 0.71, p = 0.05) was positively correlated, whereas c-Fos expression in PL and PVN was negatively correlated ($r_{(8)}$ = -0.91, p < 0.01); positive correlations between PL and CA1 ($r_{(8)}$ = 0.67, p = 0.07) and CA1 and CA3 ($r_{(9)}$ = 0.59, p = 0.10) approached significance. Thus, acute stress alone alters the pattern of activity across the corticolimbic brain regions examined, decreasing the number and strength of associations between regions in both male and female rats. Again, however, the pattern of correlated activity across regions varies between males and females.

In CRS-EPS male rats, c-Fos expression in PL and IL ($r_{(11)}$ = 0.70, p = 0.02), CA1 and CA3 ($r_{(12)}$ = 0.67, p = 0.02), CA1 and BLA ($r_{(12)}$ = 0.69, p = 0.01), CA3 and BLA ($r_{(12)}$ = 0.66, p = 0.02), and OFC and BLA ($r_{(12)}$ = 0.62, p = 0.03) were significantly and positively correlated. In CRS-EPS female rats, c-Fos expression in PL and IL ($r_{(8)}$ = 0.91, p < 0.01) were significantly and positively correlated, while c-Fos expression in DG was significantly and negatively correlated with activation in both PL ($r_{(8)}$ = -0.76, p = 0.03) and IL ($r_{(8)}$ = -0.91, p < 0.01). A positive correlation between activation in CA1-CA3 and approached significance ($r_{(9)}$ = 0.58, p = 0.10). Thus, in both males and females, chronic stress produced different patterns of correlated activity across the corticolimbic regions examined relative to acute stress. Furthermore, these patterns were markedly different in CRS-EPS males and females.

In CRS-Rest-EPS male rats, only one correlation approached significance (CA1-BLA, $r_{(9)}$ = -0.60, p = 0.09). In contrast, in CRS-Rest-EPS female rats, c-Fos expression in PL and PVN ($r_{(5)}$ = 0.87, p = 0.05) and in IL and DG ($r_{(6)}$ = 0.87, p = 0.02) were significantly and positively correlated. Positive correlations between OFC and CA3 ($r_{(8)}$ = 0.65, p = 0.08) and OFC-DG ($r_{(8)}$ = 0.62, p = 0.10) approached significance, as did a negative correlation between PVN and CA3 ($r_{(7)}$ = -0.69, p = 0.09). Thus, EPS-induced c-Fos expression in chronically stressed male rats given a rest period showed little association across the corticolimbic regions examined, in chronically stressed female rats given a rest period, EPS produced an increase in correlated activation across these areas.

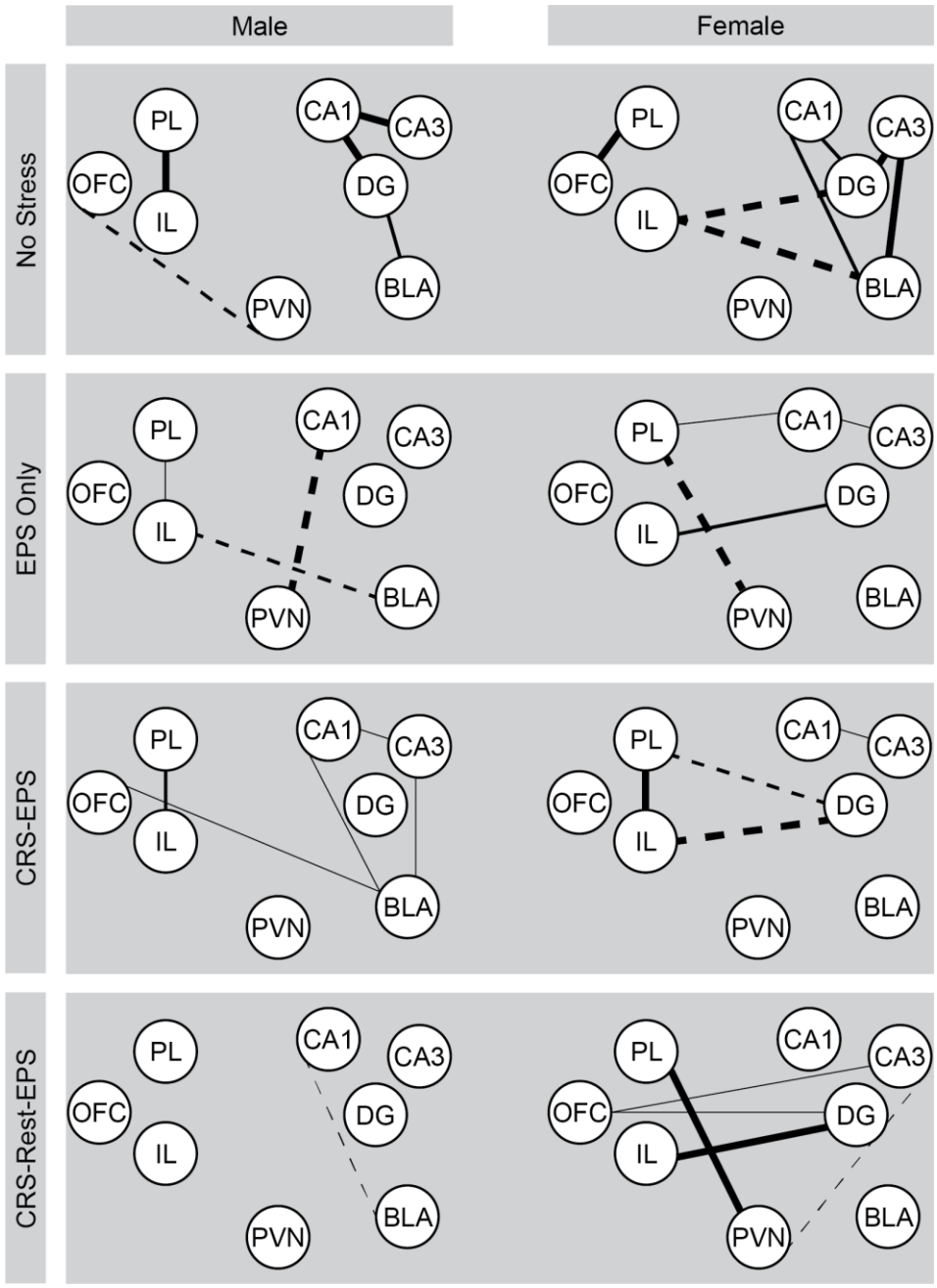


Figure 3.7. Summary of correlational analyses of c-Fos expression across corticolimbic brain regions. Spearman correlations were used to assess potential associations in EPS-induced neuronal activation between OFC, PL, IL, PVN, CA1, CA3, DG, and BLA in males and females. Positive and negative correlations are represented by solid and dashed lines, respectively. The strength of each correlation is represented by line weight (——, — —, $r = \pm 0.81 – 1.0$; ——, — —, $r = \pm 0.71 – 0.80$; —, − −, $r = \pm 0.60 – 0.70$).

Discussion

These results demonstrate sex differences in the modulation of acute stress-induced cellular activation by chronic stress with and without a rest period in a number of corticolimbic brain regions (see Table 3.1). In chronically stressed males not given a rest period, acute stress-induced activation was blunted in PL, IL, CA1, DG, the PVN, with a similar but nonsignificant tendency in the BLA. This blunted response was also present in PL, CA1, and DG after a 7-day rest period, whereas acute stress-induced activation in IL, BLA, and PVN was comparable to that seen in EPS-only males. In contrast, in female rats, prior chronic stress resulted in blunted acute stress-induced activation only in OFC. Notably, following a rest period, chronically stressed female rats had elevated c-Fos expression in BLA and the PVN, with a similar but nonsignificant tendency present in the DG following a novel acute stressor.

Table 3.1. Effects of chronic stress without and with a rest period on acute stress-induced c-Fos expression in corticolimbic brain regions.

Sex:	**Male**		**Female**	
Stress:	**No Rest**	**Rest**	**No Rest**	**Rest**
Prelimbic Cortex	↓	↓	-	-
Infralimbic Cortex	↓	-	-	-
Orbitofrontal Cortex	-	↑	↓	↓
CA1	↓	↓	-	-
CA3	-	-	-	-
Dentate Gyrus	↓	↓	-	↑
Basolateral Amygdala	↓	-	-	↑
Periventricular Nucleus	↓	↓	-	↑

Note: ↑ (green) indicates increased c-Fos expression compared to EPS Only rats of same sex; ↓ (red) indicates decreased c-Fos expression compared to EPS Only rats of same sex; darker shading, $p \leq 0.05$; lighter shading, $p \leq 0.09$.

<u>Region-specific, persistent reduction of novel stress-induced c-Fos expression in chronically stressed male rats with and without a rest period.</u>

Interest in the plasticity of adult rodent brain in the aftermath of chronic stress is growing. Conrad and colleagues (1999) provided the first evidence suggesting that chronic stress-induced

changes were not permanent, demonstrating that in males, chronic stress-induced dendritic retraction in hippocampal CA3 pyramidal neurons was no longer present 10 days after the cessation of chronic stress. Further, chronic stress-induced deficits in spatial memory are also ameliorated following a post-stress rest period (Sousa et al., 2000). In contrast to the hippocampus, chronic stress leads to increased dendritic arborization in BLA (Vyas et al., 2002; Vyas et al., 2003; Vyas et al., 2004; Johnson et al., 2009), although this effect may depend on stressor intensity and/or duration, as others have found dendritic retraction (Grillo et al., 2015). In the case of dendritic outgrowth, a 21-day rest period is not sufficient to reverse chronic stress-induced changes (Vyas et al., 2004). In mPFC, chronic stress-induced retraction is ameliorated following a rest period in males (Radley et al., 2005; Moench and Wellman, 2017), suggesting that the reversibility of chronic stress-induced changes in dendritic architecture is region-specific. We recently showed that, in males, the process of post-stress dendritic remodeling in mPFC is dynamic – chronically stressed male rats given a 7-day rest period exhibit dendritic outgrowth beyond unstressed lengths (Moench and Wellman, 2017). This finding raises the possibility that a novel stress challenge during the post-stress rest period could have important functional implications for mPFC and other stress-sensitive brain regions. The present findings provide further support for this notion.

Previous studies have found reduced stress-evoked c-*fos* mRNA expression in a number of brain regions involved in stress regulation following exposure to repeated homotypic stress (e.g., Melia et al., 1994; Campeau et al., 2002; Girotti et al., 2006). However, few studies have examined the effects of prior chronic stress on heterotypic stress-induced neuronal activation. Those that have reveal conflicting findings. For example, male rats exposed to chronic cold stress have greater c-Fos induction in the periventricular thalamus and amygdala following a restraint challenge (Bhatnagar and Dallman, 1998). Similar results have been found in male rats exposed to chronic restraint stress prior to an acute social defeat stressor (Chung et al., 2000). In contrast, chronic variable stress followed by exposure to a novel environment results in reduced c-*fos*

mRNA expression in males in a number of cortical and non-cortical regions, including lateral septum, lateral hypothalamus, anterior cingulate cortex, PVN, and PL (Ostrander et al., 2009). A similar finding was reported in BLA of male rats exposed to a novel stress challenge one day following the cessation of CRS (Reznikov et al., 2008). The reason for discrepant findings among other studies is unclear but may reflect differences in the intensity and/or salience of the novel stress challenge in relation to the chronic stress paradigm. On the other hand, differences in *c-fos* mRNA may not directly reflect changes in c-Fos protein expression. For instance, repeated, severe immobilization produced changes in *c-fos* mRNA but not c-Fos immunostaining in several brain regions (Ons et al., 2010). However, it is unknown if this is also the case following a heterotypic stress challenge such as the paradigm used here.

Indeed, our findings of reduced c-Fos expression in multiple corticolimbic brain regions in response to a novel stress challenge following chronic restraint stress are in agreement with Ostrander and colleagues (2009) and Reznikov and colleagues (2009), and suggest that changes in *c-fos* mRNA parallel changes in the protein following a heterotypic acute stressor. This regional specificity in chronic stress modulation of cellular excitability in response to an immediate novel stress challenge may indicate dynamic underlying neurobiological changes that differ across regions and may aid in stress adaptation. Together, these data indicated that in males, chronic stress produces a prolonged period of neuronal hyporesponsivity that varies by region and post-chronic stress time point. Further, they suggest, as recently argued by Ortiz and Conrad (Ortiz and Conrad, 2018), that post-stress changes in corticolimbic regions do not constitute a recovery—that is, a return to baseline—but rather reflect a new functional state for these structures, which is distinct from either the unstressed or stressed condition.

In females, chronic stress without a rest period does not modulate novel stress-induced activation, but novel stress-induced activation is potentiated following rest.

In contrast to the effects of chronic stress in males, in females, chronic stress largely did not modulate neuronal activity in response to a heterotypic acute stressor. The notable exception here is the reduction found in c-Fos expression in OFC, which was one of just two regions we examined in which chronic stress only marginally altered c-Fos expression in male rats.

This is the first study to our knowledge to examine prior chronic stress effects on neuronal activity following a heterotypic stress challenge in adult females. Notwithstanding, our results align with a growing body of literature suggesting that chronic stress produces few neurobiological and behavioral changes in adult female rodents. Indeed, chronic stress does not disrupt behaviors mediated by hippocampus (Bowman et al., 2003) or mPFC (Wei et al., 2014; Snyder et al., 2015), and does not produce dendritic retraction in these regions (Galea et al., 1997; Moench and Wellman, 2017). These studies and others have led some to conclude that female rats are resilient to chronic stress paradigms, especially with regards to cognitive changes following stress (Luine et al., 2017). Our findings here that, across many corticolimbic brain regions that are involved in cognitive processing, chronic stress does not modulate novel acute stress-induced neuronal activity could be interpreted as largely consistent with this notion. However, given that chronic stress did result in blunted neuronal activation in OFC of females, it is likely that females are not resilient to the effects of chronic stress, but instead show a different pattern of changes following chronic stress compared to males.

Indeed, chronically stressed female rats given a rest period showed *enhanced* c-Fos expression following a novel acute stressor, most notably in the PVN and BLA. Although it is possible that we overestimated the number of double-labeled cells due to nonspecific binding of the CRH antibody, the percentage of CRH-immunoreactive cells that were c-Fos-positive was in line with previous findings (e.g., Romeo et al., 2006). Thus, given that many CRH-immunoreactive cells in the PVN were c-Fos positive, and the possibility that projections from the BLA may act in

a feed-forward manner to facilitate the PVN response to stress (Bhatnagar et al., 2004), this pattern of findings could reflect HPA axis hyperresponsivity to novel stress during the post-chronic stress rest period in female rats. Ostrander and colleagues (2009) demonstrated that reduced novel stress-induced c-*fos* expression in the PVN was associated with reduced plasma corticosterone in male rats. This suggests that changes in neuronal activation in the PVN in our model of two-hit stress likely result in altered neuroendocrine functioning. Although it is beyond the scope of the current study, future work should determine if these findings in females represent an exaggerated HPA response to a novel stressor in previously stressed female rats, and if so, what behavioral and neurobiological ramifications might result from such a response. Further, these data support the notion that females are not 'resilient' to the effects of chronic stress. Instead, it appears that male and female rats respond differently to chronic stress, and that these differences result in sex-specific responses to future stress challenges.

Inter-region correlations suggest circuit-level changes in neuronal activation in following a novel stress challenge.

Our correlational analyses of c-Fos across brain regions revealed striking patterns of associations and dissociations between regions in chronically stressed male and female rats without and with a rest period compared to stress-naïve rats. These patterns suggest that there are sex-specific circuit-level changes in the neural response to an acute stress challenge following both chronic stress and a rest period, and highlight the unique pattern of activation across brain regions after EPS stress in CRS-Rest females. For instance, in females, chronic stress appears to produce a striking "uncoupling" of BLA from PFC and hippocampal areas. Interestingly, a similar pattern of uncoupling has been found in healthy adults following prolonged periods of occupational stress (Liston et al., 2009b; Jovanovic et al., 2011), suggesting that chronic stress may reduce functional connectivity between corticolimbic brain regions. It is interesting to speculate that this might result in increased effects of subsequent novel stressor on anxiety-like

and/or fear behavior, perhaps reflecting less inhibitory control from PFC to BLA. Similarly, the altered relationship between BLA and hippocampus could have implications for contextual fear, discrimination, or generalization. Recent work from our lab has shown that there are also sex differences in microglia activation across corticolimbic brain regions, with the number, strength, and direction of correlations between brain regions differing following either acute or chronic stress (Bollinger et al., 2017). While the ramifications of these differing patterns of inter-regional associations are presently unclear, they nonetheless highlight the need to examine sex differences in the effects of stress not only in single regions of interest, but also at the level of potential circuit-wide changes that might contribute to stress adaptation or maladaptation. Indeed, several recent reviews have highlighted the growing necessity for neuroscience research to move beyond single neuronal populations, and even individual brain regions (Yuste, 2015; Krakauer et al., 2017), to truly elucidate how neural circuits give rise to healthy and pathological behavioral states.

Conclusions

These data demonstrate that chronic stress modulates novel acute stress-induced c-Fos expression in a sex-, region-, and rest-dependent manner. Notably, while chronic stress tended to produce immediate reductions in acute stress-induced neuronal activity in a number of corticolimbic brain regions in male rats, we found little modulation of neuronal activity in several corticolimbic regions in female rats. An important exception to this pattern: following a post-stress rest period, chronically stressed female rats had increased activation in the BLA and PVN, which could contribute to an exaggerated HPA axis response to a novel stress challenge. These results suggest that the post-stress rest period may give rise to sex-specific mechanisms underlying stress adaptation and underscore the necessity of understanding not only the immediate consequences of chronic stress, but also the lasting sequelae of stress, and how these changes may modulate the brain's response to future perturbations.

Chapter 4:

Sex-Specific Effects of Two-Hit Stress on Extradimensional Set-Shifting in Rats.

In Chapter 2, I showed that male and female rats show differential dendritic remodeling in the days following chronic stress. In chronically stressed males, not only was chronic stress-induced dendritic retraction ameliorated, but apical dendrites were longer than those in unstressed males following a 7 day rest period. In contrast, I found minimal dendritic remodeling in females during the post-stress time. In Chapter 3, I went on to show that this sex-specific dendritic remodeling might contribute to functional differences in medial prefrontal cortex of chronically stressed males and females following a post-chronic stress rest period. In chronically stressed male rats, neuronal activation in response to a novel stress challenge was blunted in the prelimbic subregion of medial prefrontal cortex both on the day following chronic stress and following a 7 day rest period. In contrast, prior exposure to chronic stress did not alter acute stress-induced neuronal activation in prelimbic cortex in females. Together, these findings suggest that two-hit stress results in sex-specific functional changes in medial prefrontal cortex. If this is the case, there may be differences in the effects of two-hit stress on behaviors mediated by medial prefrontal cortex.

Cognitive flexibility – the ability to shift behavioral or cognitive responses using feedback from the environment (Berg, 1948) – is a behavior mediated by prefrontal cortex that can be disrupted in individuals with stress-related disorders (Rock et al., 2014; Scott et al., 2015). Attentional set-shifting tasks measure cognitive flexibility by assessing the ability to shift responses when contingencies change, thus requiring attentional disengagement from one stimulus and attentional reengagement in a different stimulus. These shifts include reversals, in which a previously unrewarded stimulus becomes the rewarded stimulus; intradimensional shifts, in which a new rewarded stimulus is within the same stimulus modality as those previously

rewarded; and extradimensional shifts, in which the new rewarded stimulus is in a different stimulus modality from those that were previously rewarded. Notably, deficits in extradimensional shifts are correlated with decreased signal change in dorsomedial PFC in individuals with depression (Heinzel et al., 2010), suggesting that dysfunction of this region could lead to impaired cognitive flexibility in individuals with stress-linked disorders. Behavioral flexibility can also be measured in rodents using an attentional set-shifting task (Birrell and Brown, 2000). Like tasks that measure cognitive flexibility in humans, this task was developed to include reversal learning, intradimensional shifts, and extradimensional shifts. Lesion studies have shown that in rodents, these shifts are mediated by orbitofrontal cortex, prelimbic cortex, and anterior cingulate cortex, respectively (Birrell and Brown, 2000; McAlonan and Brown, 2003; Ng et al., 2007).

In male rats, chronic stress tends to preferentially disrupt extradimensional shifts (Nikiforuk and Popik, 2011, 2013, 2014; Jett et al., 2017), although reversal learning can also be affected, especially in cases where chronic physical stressors are used (e.g., chronic intermittent cold stress; Danet et al., 2010; Wallace et al., 2014a). In contrast, sub-chronic social defeat stress does not alter performance on any stage of the set-shifting task in females (Snyder et al., 2015), although one study found that 30 minutes of daily restraint for 5 days disrupted operant set-shifting in females (Grafe et al., 2017). The findings of the former are in agreement with other studies demonstrating a lack of stress-induced deficits in tasks mediated by medial prefrontal cortex, including both spatial and non-spatial working memory tasks (Bowman et al., 2001; Bowman et al., 2003; Wei et al., 2014). To date, no studies have examined the effects of more chronic stressors on set-shifting performance in females.

It is currently unknown whether chronic stress-induced deficits in extradimensional shifting in males persists over time, or whether chronic stress alters performance on any phase in the attentional set-shifting task in females. Further, given the sex-specific changes in novel stress-induced neuronal activation across corticolimbic brain regions (Chapter 3; Moench et al., 2019), it is possible that exposure to a novel acute stressor during the post-chronic stress rest period

could result in sex-specific behavioral changes in behavioral flexibility. Here, I examine if chronic stress-induced deficits in extradimensional shifting in males are ameliorated following a 7-day rest period and characterize chronic stress effects on attentional set-shifting in females. I also determine how set-shifting behavior changes after two-hit stress in both males and females.

Materials and Methods

Subjects and Stressors

Male and female Sprague Dawley rats (approximately 10 weeks of age at start; Envigo, Indianapolis, IN) were individually housed in standard laboratory cages (48 cm × 20 cm × 26 cm), with ambient temperature 23-25 °C, free access to water, and a 12:12 light/dark cycle (lights on at 0800 h). To motivate rats for behavioral testing, body weights were reduced to 85% of free-feeding weight. All procedures were conducted between 8:00 am and 6:00 pm, were in accordance with NIH Guidelines, and were approved by Indiana University's IACUC.

Chronic stress consisted of daily restraint (CRS; 3 h/day, 10 d). Rats were weighed daily throughout the stress procedure. Immediately after weighing, No CRS rats were returned to their home cages and left undisturbed in a separate room. CRS rats were placed in semi-cylindrical restrainers (male, 16 cm length × 6.5 cm width × 5 cm height; female, 15 cm length × 6 cm width × 4.5 cm height, modified so the tail piece locks into place; Braintree Scientific, Braintree, MA) in their home cages, with the time of restraint unpredictably varied over the light cycle. This manipulation produces significant increases in plasma corticosterone levels (Cook and Wellman, 2004). Elevated platform stress (EPS) consisted of placing each rat individually on a small platform (12 cm × 12 cm) elevated 90 cm off the ground for 30 min in a brightly lit room as previously described (Xu et al., 1997; Maroun and Richter-Levin, 2003; Maroun et al., 2013). For this study, a mixed between- and within-subjects design was used. Rats were assigned to one of three stress conditions, all of which underwent behavioral testing at two timepoints (See Fig. 4.1 for experimental design and timeline). The first group was not exposed to chronic stress prior to

the first test session. Rats in this group were then exposed to EPS six days later, with the second test session on the day after EPS (No CRS-EPS; n = 9 male, 9 female). The second group underwent chronic stress followed by EPS on the day after chronic stress ended. The first test session for this group occurred the day after EPS and the second test occurred after a six-day rest period (CRS-EPS-Rest; n = 8 male, 10 female). The third group underwent chronic stress and was tested for the first time on the day after chronic stress. Rats in this group were then exposed to EPS six days later and the second test session occurred on the day after EPS (CRS-Rest-EPS; n = 8 male, 13 female).

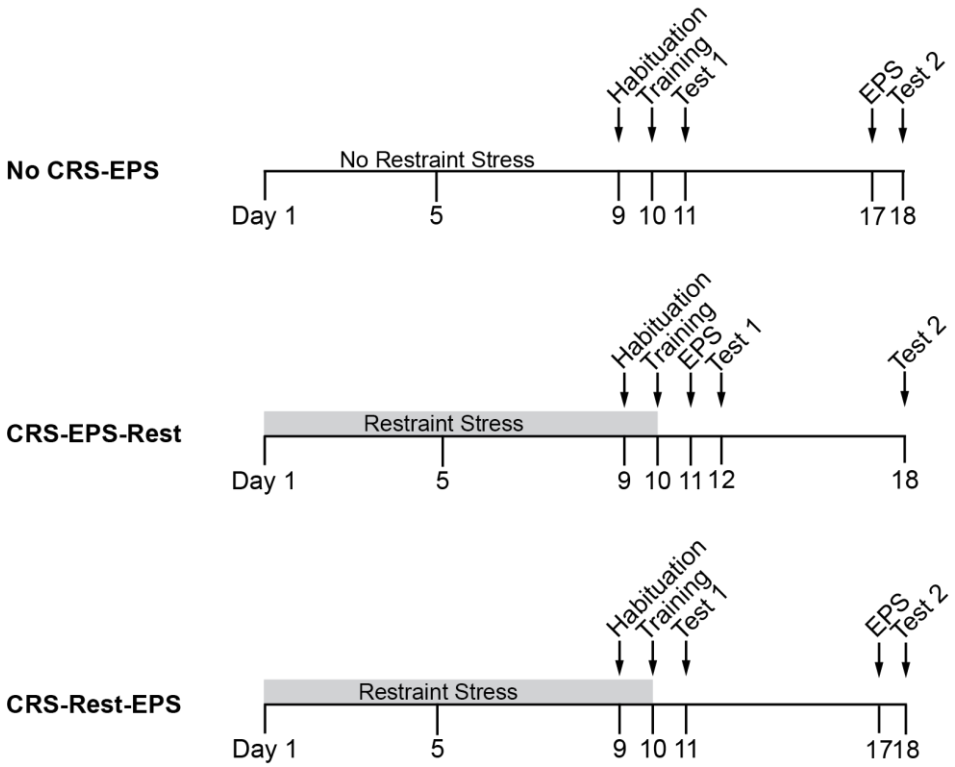


Figure 4.1. Experimental design and timeline. (*Top*) Rats in this condition were not exposed to chronic restraint stress prior to the first testing session. One day prior to the second test session, rats were exposed to EPS. (*Middle*) Rats in this condition underwent chronic restraint stress and were then exposed to EPS on the day prior to the first test session. Test session two occurred following a rest period. (*Bottom*) Rats in this condition underwent chronic restraint stress followed by the first test session after the cessation of chronic stress. Following a rest period, rats were exposed to EPS on the day prior to the second test session.

Attentional Set-Shifting Test

Rats were trained on an attentional set-shifting task as previously described (Birrell and Brown, 2000; Lapiz-Bluhm et al., 2008). All training and testing took place under dimly lit conditions prior to stress on experimental Days 9 and 10, and before rats were given their daily food ration. This task consisted of training rats to dig in small terracotta pots (2.5 inches height × 3 inches width) for a food reward (BioServ pellets; Holton Industries, Frenchtown, NJ). The pot rims could be scented and/or covered with textures. Thus, pots could differ along two stimulus dimensions: odor and texture (see Table 4.1 for stimuli used). The testing apparatus was a rectangular black Plexiglas arena (24 in length × 16 in width ×8 in height) with a panel to divide one-third of the arena into two sections, in which pots were placed. A removable divider separated the start area from these two sections.

Habituation. On Day 9 of restraint stress (Day 9 of food restriction in No CRS rats) rats were habituated to the apparatus and trained to dig in unaltered pots. During habituation, unscented pots filled with digging medium (bedding) were place in both testing sections with sucrose pellets placed on top. Rats were allowed to move about the arena freely. When both sucrose pellets were retrieved, rats were placed back into the start area, pellets were replenished, and rats were again allowed to move about the arena freely. Sucrose pellets were gradually buried further into the digging medium in order to shape digging behavior. This continued until rats reliably retrieved both sugar pellets. Rats that did not develop digging behavior did not go on to simple discrimination training. This was the case for 4 rats (male, n = 2, CRS-EPS-Rest; female, n = 2, CRS-Rest-EPS).

Table 4.1. Stimuli used in the AST.

	Training Pair	Test Pair 1	Test Pair 2	Test Pair 3
Odor	Cinnamon/Oregano	Blueberry/Lavender	Ginger/Nutmeg	Cherry/Lemon
Texture	Bubble wrap/Towel	Twine/Pebbles	Wood Balls/Pom poms	Glass tiles/Beads

Simple Discrimination Training. On Day 10 of restraint (Day 10 of food restriction in No CRS rats) rats were trained on both an odor and texture simple discrimination in which two pots were presented (e.g., cinnamon versus oregano or the texture of bubble wrap versus that of pipe cleaner) but only one was baited. A trial was initiated when the removable start area panel was lifted, and rats were given access to both pots. During the first four trials a dig first in the unbaited pot was recorded as an error but the rat was then permitted to dig in the baited pot to retrieve the reward. Beginning on the fifth trial, if the rat dug in the unbaited pot first, an error was recorded, and the trial was ended by placing the rat back in the start area. This continued until six consecutive correct trials were recorded. Digging was defined as the displacement of digging medium by either the nose or the nose and front paws. The stimuli used during training were not used again during testing.

Testing. On test days (days 11 or 12 and 18) rats were tested on a series of increasingly difficult discriminations (see Table 4.2 for an example protocol). Each test session began with a simple discrimination (SD) where pots differed along only one dimension. For the compound discrimination, the second dimension was added, but the correct and incorrect stimuli from the SD phase remained constant. For the first reversal (Rev1), the stimuli and correct dimension were unchanged from the CD stage, but the previously incorrect stimulus was rewarded. For the intradimensional shift (IDS), all the stimuli were replaced, but the correct dimension from the prior three phases remain constant. A second reversal (Rev2) followed the IDS in an identical manner to Rev1. Finally, for the extradimensional shift (EDS), all the stimuli were replaced, *and* the correct dimension was shifted to the previously incorrect dimension. For rats that began testing with odor as the correct dimension, texture became the correct dimension in the EDS; for rats that began testing with texture as the correct dimension, odor became the correct dimension. The order of the EDS was counterbalanced between groups and within animals between testing sessions. It has been demonstrated that this set-shifting task can be used in a within-subjects design without significant facilitation of performance in the second testing session (Wallace et al., 2014b).

During each stage of training and testing, trials continued until the rat made six consecutive correct choices, and trials to criterion were recorded. Three rats (male, n = 1, No CRS-EPS; female, n = 2, CRS-Rest-EPS) did not finish testing during the first test session, but data were collected from these rats during the second test session. One rat did not perform the task during the second test session (male, No CRS-EPS) but data from the first test session were included in analyses. The final n's per group for Test 1 are: No CRS-EPS, 9 male, 9 female; CRS-EPS-Rest, 8 male, 9 female; CRS-Rest-EPS, 8 male, 10 female. The final n's per group for Test 2 are: No CRS-EPS, 8 male, 9 female; CRS-EPS-Rest, 8 male, 9 female; CRS-Rest-EPS, 8 male, 10 female.

Table 4.2. Representative example of stimulus pairing on the AST.

Discrimination Stage	Dimensions		Example Combinations	
	Relevant	Irrelevant	(+)	(-)
Simple (SD)	Odor		**Blueberry**	Lavender
Compound (CD)	Odor	Texture	**Blueberry**/Twine **Blueberry**/Pebbles	Lavender/Pebbles Lavender/Twine
Reversal 1 (Rev1)	Odor	Texture	**Lavender**/Pebbles **Lavender**/Twine	Blueberry/Twine Blueberry/Pebbles
Intradimensional Shift (IDS)	Odor	Texture	**Ginge**r/Wood Balls **Ginger**/Pom Poms	Nutmeg/Pom Poms Nutmeg/Wood Balls
Reversal 2 (Rev2)	Odor	Texture	**Nutmeg**/Pom Poms **Nutmeg**/Wood Balls	Ginger/Wood Balls Ginger/Pom Poms
Extradimensional Shift (EDS)	Texture	Odor	Lemon/**Beads** Cherry/**Beads**	Cherry/Tiles Lemon/Tiles

Characterization of Estrous Phase

To examine the potential effects of ovarian hormones on behavioral performance, vaginal lavages were performed on each testing day. After the first test session, lavages were performed on awake rats in a separate room from the testing room. After the second test session, lavages were performed immediately after rats were anesthetized for tissue collection. In both cases, exfoliate cytology was examined promptly under light microscopy to determine estrous phase as previously described (Garrett and Wellman, 2009).

Statistical Analyses

Data were first analyzed via a four-way repeated measures ANOVA (sex x stress condition x testing stage x testing session; stage and session as the repeating measures). To minimize the number of pairwise comparisons, the following strategy was use. Following the initial omnibus test, planned comparisons were used to determine the source of significant interactions. First, to determine if there was a basic sex difference in performance on any stage of the task, male and female No CRS-EPS (Test 1) were compared using a one-way ANOVA. Following this initial comparison, data from males and females were analyzed separately. One-way ANOVAs were used to compare groups within the same test session. Significant effects were followed by Fisher's protected LSD *post hoc* comparisons. Paired t-tests were used to compare data within each stress group across test sessions.

Results

Basal Sex Differences in Set-Shifting Performance

The four-way repeated measures ANOVA revealed main effects of test stage ($F_{(4, 172)}$ = 17.26, $p < 0.001$), and stress ($F_{(2, 43)} = 8.92$, $p = 0.001$), but not test session ($F_{(1, 43)} = 0.01$, n.s.) or sex ($F_{(1, 43)} = 0.01$, n.s.). Several two-way interactions were significant, including test stage by sex ($F_{(4, 172)} = 5.22$, $p = 0.001$), test stage by stress ($F_{(8, 172)} = 9.54$, $p < 0.001$), test session by sex ($F_{(1, 43)} = 51.97$, $p < 0.001$), and sex by stress ($F_{(2, 43)} = 3.21$, $p = 0.05$), but not test session by stress ($F_{(2, 43)} = 2.41$, n.s.) or test stage by test session ($F_{(4, 172)} = 2.40$, n.s.). All three-way interactions were significant, including test stage by sex by stress ($F_{(8, 172)} = 3.49$, $p = 0.001$), test session by sex by stress ($F_{(2, 43)} = 19.73$, $p < 0.001$), test stage by test session by sex ($F_{(4, 172)} = 28.38$, $p < 0.001$), and test stage by test session by stress ($F_{(8, 172)} = 2.61$, $p = 0.01$). Finally, the four-way interaction (test stage by test session by sex by stress) was also significant ($F_{(8, 172)} = 10.85$, $p < 0.001$).

Trials to criterion on the first test session differed between No CRS-EPS males and females on the extradimensional shift (Fig. 4.2; $F_{(1, 16)} = 4.98$, $p = 0.04$), such that females required fewer trials to complete this stage. Performance on all other stages was comparable (Fs ≤ 0.13, all n.s.).

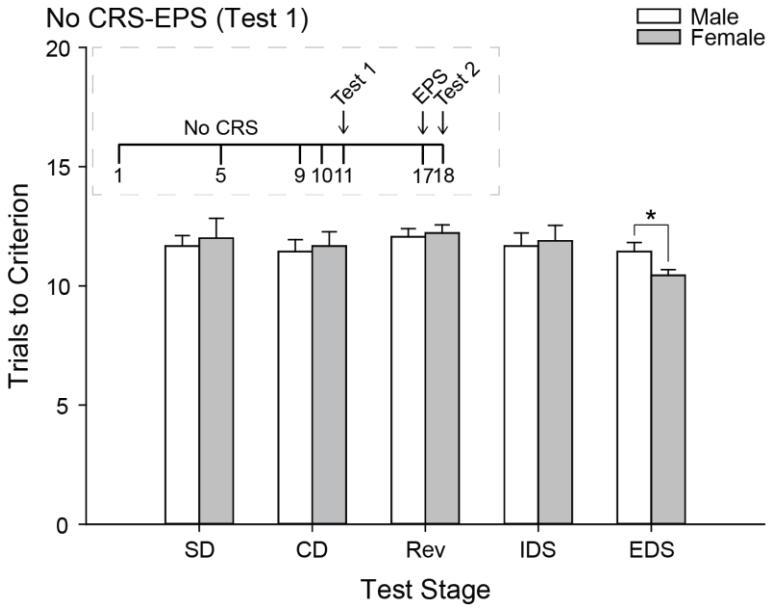


Figure 4.2. Basal sex differences in the attentional set-shifting test. For EDS, unstressed male rats required more trials to reach criterion than unstressed females. * p < 0.05. Error bars represent SEM.

Stress Effects on Set-Shifting in Males

The effects of chronic stress on set-shifting in males were examined using a one-way ANOVA comparing trials to criterion on the first test session between the three groups. Trials to criterion did not differ on any stage of testing except for the extradimensional shift (Fig. 4.3 A; EDS, $F_{(2, 21)} = 27.14$, $p < 0.001$; all other stages, Fs ≤ 2.22, all n.s.). *Post hoc* analyses revealed that both CRS-EPS-Rest ($p < 0.001$) and CRS-Rest-EPS ($p < 0.001$) males required more trials to complete the extradimensional shift compared to No CRS-EPS, indicating that chronic stress preferentially disrupts performance on this stage.

Paired t-tests were then used to compare performance on each test stage within each group. Trials to criterion did not differ from test session one to test session two in No CRS-EPS male rats (Fig. 4.3 B; -0.33 ≤ ts ≤ 1.04, all n.s.), indicating that acute stress did not alter

performance on any stage of the task. For CRS-EPS-Rest males, trials to criterion differed only on the extradimensional shift (Fig. 4.3 C; $t_{(7)} = 8.37$, $p < 0.001$) such that fewer trials to criterion were required on the second test session. Thus, although chronic stress disrupted initial extradimensional set-shifting, this deficit was no longer present after a rest period. For CRS-Rest-EPS males, trials to criterion differed on both the intradimensional (Fig 4.3 D; $t_{(6)} = 2.57$, $p = 0.04$) and extradimensional stages ($t_{(6)} = 8.37$, $p = 0.001$), such that fewer trials to criterion were needed during the second test session. Performance did not differ on any other test stage ($-0.57 \leq t\text{s} \leq 1.00$, all n.s.). Thus, although chronic stress initially disrupts extradimensional set-shifting, this deficit was no longer present after exposure to a novel acute stressor, whereas intradimensional set-shifting performance is facilitated in these males.

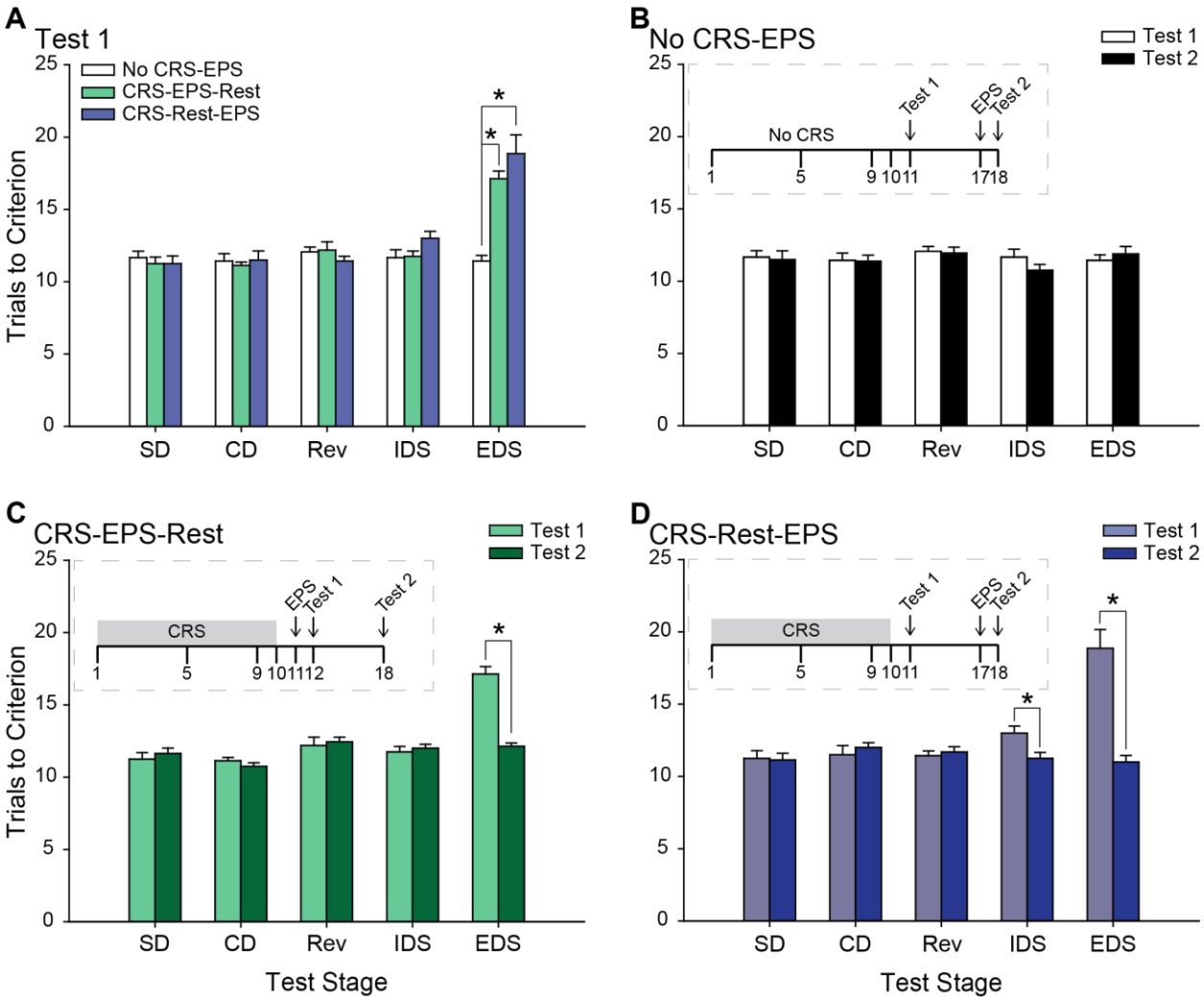


Figure 4.3. The effects of stress on attentional set-shifting in male rats. (A) Chronic stress and chronic stress followed one day later by EPS impairs performance on the extradimensional shift compared to unstressed males. (B) EPS alone does not alter performance on any phase of the test in previously unstressed rats. (C) Extradimensional shifting is no longer impaired following a rest period in male rats that were first tested immediately following CRS and EPS. (D) Extradimensional shifting is not impaired in male rats who underwent CRS, were given a rest period, and then exposed to EPS. Intradimensional shifting is also facilitated in the second test session in these males. Error bars represent SEM.

Stress Effects on Set-Shifting in Females

The effects of chronic stress on set-shifting in females were examined using a one-way ANOVA comparing trials to criterion on the first test session between the three groups. Trials to criterion did not differ on any stage of testing (Fig. 4.4 A; Fs ≤ 1.51, all n.s.). Thus, in females, chronic stress does not alter set-shifting performance.

Paired t-tests were then used to compare performance on each test stage within each group. Trials to criterion did not differ from test session one to test session two in No CRS-EPS female rats (Fig. 4.4 B; -1.90 ≤ ts ≤ 0.73, all n.s.), indicating that acute stress did not alter performance on any stage of the task. For CRS-EPS-Rest females, trials to criterion did not differ on any stage of the task between test session one and two (Fig. 4.4 C; -0.89 ≤ ts ≤ 0.37, all n.s.), indicating that the presence of a post-chronic stress rest period does alter set-shifting performance. For CRS-Rest-EPS females, trials to criterion differed between test session one and two on both the simple discrimination (Fig 4.4 D; $t_{(9)} = -2.53$, $p = 0.03$) and extradimensional ($t_{(7)} = -6.60$, $p < 0.001$) stages such that more trials to criterion were needed on both of these stages during the second test session. Thus, although chronic stress does not alter performance on the set-shifting task, exposure to a novel acute stressor following a rest period results in deficits in both simple discrimination and extradimensional set-shifting.

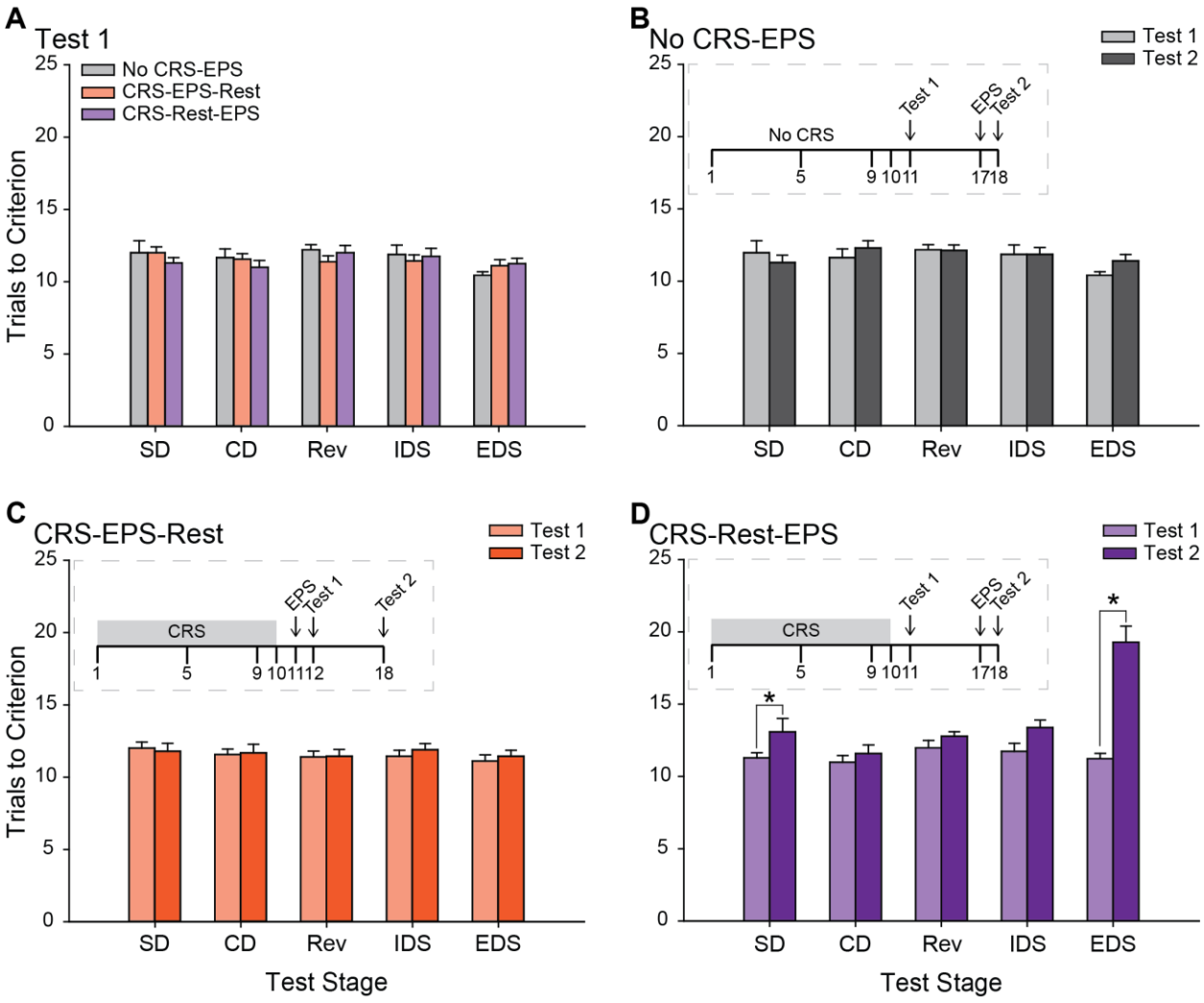


Figure 4.4. The effects of stress on attentional set-shifting in female rats. (A) Chronic stress and chronic stress followed one day later by EPS do not impair performance on any phase of the test compared to unstressed females. (B) EPS alone does not alter performance on any phase of the test in previously unstressed rats. (C) Task performance before and after a rest period is unchanged in females exposed to chronic stress followed one day later by EPS. (D) Extradimensional shifting is impaired in female rats who underwent CRS, were given a rest period, and then exposed to EPS. Trials to criterion are also increased in the simple discrimination phase in these females. Error bars represent SEM.

Discussion

The data presented in this chapter show that there are sex-specific effects of both chronic and two-hit stress on attentional set-shifting. In males, chronic stress impaired performance in extradimensional set-shifting. This deficit was ameliorated following a 7 day rest period. In contrast, chronic stress alone did not alter attentional set-shifting in females. Instead, 7 days after chronic stress, exposure to a second stressor impaired performance on the extradimensional shift in females. This effect was absent in male rats.

In males, chronic stress-induced deficits in extradimensional set-shifting are reversible.

Previous studies have shown that chronic stress impairs performance on a number of tasks that are mediated by mPFC in male rats. For example, 21 days of restraint stress results in deficits in temporal order working memory (Wei et al., 2014) and object recognition (Bowman et al., 2003). A number of studies have also shown that chronic stress disrupts extradimensional set-shifting in males (Liston et al., 2006; Nikiforuk and Popik, 2011, 2013, 2014; Jett et al., 2017). The data presented here agree with these previous findings in that males exposed only to chronic stress or to chronic stress followed one day later by acute stress have deficits in the attentional set-shifting task that are specific to the extradimensional shift. Given that extradimensional set-shifting is mPFC-dependent, it follows that mPFC of male rats is sensitive to the deleterious effects of chronic stress on the function of mPFC. What was previously unknown is whether these behavioral deficits induced by chronic stress are reversible.

The first studies to examine the reversibility of chronic stress effects on the brain and behavior came from studies of the hippocampus. Performance on hippocampus-dependent spatial working memory tasks, including the Morris Water Maze and the radial arm maze, are also disrupted following chronic stress in males (Luine et al., 1994; Conrad et al., 1996; Sousa et al., 2000; Bowman et al., 2003; Hoffman et al., 2011; McFadden et al., 2011). The first evidence that these behavioral deficits were reversible following the cessation of chronic stress came from Luine

and colleagues (1994), who demonstrated that chronic stress-induced deficits in the radial arm maze were not found in rats that were tested 18 days after chronic stress ended. Since this initial finding, many studies have shown that chronic stress-induced deficits in hippocampus-dependent tasks are reversible (Sousa et al., 2000; Hoffman et al., 2011; McFadden et al., 2011; Ortiz et al., 2014; Ortiz et al., 2015; Ortiz et al., 2018).

Here, I show that deficits in extradimensional set-shifting induced by chronic stress are also reversible following a 7-day no-stress rest period, which agrees with my previous study demonstrating that chronic stress-induced dendritic retraction is also ameliorated at this time point (Chpater 2; Moench and Wellman, 2017). Only one other group has investigated the lasting effects of chronic stress on attentional set-shifting. Nikiforuk and Popik (2011) have shown that 1 hour of daily restraint for 7 days results in deficits in extradimensional set-shifting that are present 3, 7, 14, and 21 days following the cessation of stress. The reasons for the discrepancy between that study and the current data are unclear, but could include differences in experimental design. Rats in the current study were habituated and trained on the final two days of restraint, whereas those in the prior study were trained after the cessation of stress. It is possible that habituating and training animals concurrent with chronic stress may produce some buffering to the chronic stress manipulation. There is some evidence that behavioral training that recruits mPFC may indeed offset some of the deleterious effects of chronic stress on other behaviors mediated by mPFC. For example, fear extinction training ameliorates chronic stress-induced deficits in attentional set-shifting (Fucich et al., 2016). Thus, it is possible that training rats during chronic stress may decrease the duration of deleterious effects of chronic stress on mPFC-dependent behaviors. In addition to differences in experimental timelines, rats in the current study were subjected to a more restrictive feeding protocol than rats in Nikiforuk and Popik (2011) who used a more mild food restriction paradigm, which may have resulted in greater motivation to complete the task. Despite the discrepant findings between the former study and the present data, the results in the current study agree with the extensive literature showing the reversibility of stress-

induced deficits in hippocampus-dependent tasks, and add to a growing literature suggesting that at least some of the deleterious effects of chronic stress in adulthood are reversible.

In females, chronic stress does not affect attentional set-shifting.

The data presented here indicate that chronic stress does not disrupt attentional set-shifting in female rats. Only two other studies have examined the effects of repeated stress paradigms on attentional set-shifting in females, and they have yielded conflicting findings. Snyder and colleagues (2015) showed that 5 days of social defeat stress did not alter reversal learning or extradimensional shifting. In contrast, Grafe and colleagues (2017) recently showed that 30 minutes of daily restraint for 5 days resulted in deficits in both reversal learning and extradimensional set-shifting in females. Together, these studies suggest that stress paradigms (social defeat versus restraint) and their chronicity (5 days versus 10 days) have different effects on the stress-sensitive brain regions recruited during the attentional set-shifting task. In the case of restraint stress, it is possible that a shorter duration of stress might result in behavioral changes in females, while these changes may be absent following habituation to a more chronic stressor.

On the other hand, a major difference between the previous studies examining set-shifting performance in females and the current study is the version of the set-shifting tasked used. Here, the original digging version of the task was used (Birrell and Brown, 2000), while both Snyder and colleagues (2015) and Grafe and colleagues (2017) used an automated, operant version of the task (Floresco et al., 2008). While this operant version of the task includes the same types of shifts as the digging version, a noticeable and important difference is the difficulty of the task. In the digging version of the task, simple discriminations are learned by rats readily, taking fewer than 15 trials, both in this study and others (Birrell and Brown, 2000; Ng et al., 2007). In contrast, rats require upwards of 50 trials to learn an initial discrimination in the operant version (Floresco et al., 2008). It is likely that this is due to the ecological relevance and salience of the stimuli used (odors and textures versus light cues), and perhaps the behavioral response (digging versus

barpressing) as well. Thus, it is hard to compare the lack of effects following chronic stress on the digging version of the task found here to previous studies using the operant version that have found both no change in performance (Snyder et al., 2015) and deficits (Grafe et al., 2017) following sub-chronic stressors.

Notwithstanding the inconsistent findings between the few studies examining the effects of stress on attentional set-shifting, the findings presented here are consistent with prior work showing no effect of chronic stress on mPFC-mediated behaviors in female rats. For example, 21 days of restraint stress does not impair temporal order working memory (Wei et al., 2014) or object recognition memory (Bowman et al., 2003). Additionally, unlike in male rats, chronic stress does not disrupt hippocampus-depend tasks in female rats, and instead often results in *enhanced* performance. For example, 10 days of chronic unpredictable stress results in better performance in the Morris Water Maze (McFadden et al., 2011). This is also the case following 21 days of restraint stress (Bowman et al., 2001). These and other studies showing no change in hippocampus-dependent behaviors following chronic stress in females (Kitraki et al., 2004; McLaughlin et al., 2010), combined with mounting evidence that chronic stress does not alter performance on mPFC-mediated tasks, suggests that female rats are somewhat resistant to the immediate effects of chronic stress on hippocampus- and mPFC-mediated behaviors.

<u>Two-hit stress disrupts extradimensional set-shifting in female, not male, rats.</u>

In Chapter 3, I showed that there are sex differences in neural activation across a number of corticolimbic brain regions, including mPFC, when chronically stressed male and female rats are exposed to a novel stressor challenge following a rest period (Moench et al., 2019). This suggests that, within this paradigm, there may be sex-specific changes in behaviors mediated by these regions. Indeed, the results of this study indicate that this is the case for extradimensional set-shifting, which is mediated by the prelimbic subregion of mPFC (Birrell and Brown, 2000). Whereas extradimensional set-shifting was unaffected by an acute stress challenge following a

post-chronic stress rest period in males, female rats had a surprising deficit that was specific to the extradimensional shift. My previous data demonstrated that males have a persistent reduction in novel stress-induced neuronal activation in prelimbic cortex, whereas females do not (Chapter 3; Moench et al., 2019). Although it is not clear how sex differences in neuronal activation in response to a novel stressor contribute to behavioral changes in the extradimensional shift on the following day, it does suggest that prelimbic cortex of males and females is responding very differently to a novel stress challenge during the post-stress time, and that there are behavioral ramifications of this sex-specific response.

Conclusions

The data presented here show that behavioral flexibility in males and females is differentially affected by chronic and "two-hit" stress. In males, chronic stress leads to a reversible deficit in extradimensional set-shifting. In contrast, in females, chronic stress does not alter behavioral flexibility as measure by the attentional set-shifting task. However, exposure to a novel stressor following a post-chronic stress rest period results in deficits in extradimensional set-shifting in female, but not male, rats.

Chapter 5:

Sex-specific changes in gene expression in prelimbic cortex following acute, chronic, and two-hit stress.

Altered glutamatergic neurotransmission in medial prefrontal cortex (mPFC) has been linked to chronic stress-induced dendritic and behavioral changes (reviewed in Popoli et al., 2011). In males, chronic stress results in downregulation of the NMDA receptor subunits NR1, NR2A, and NR2B (Lee and Goto, 2011; Wei et al., 2014; Shepard and Coutellier, 2018). These changes are associated with deficits in temporal order working memory (Wei et al., 2014). Downregulation of the AMPA receptor subunit GluR1 also has also been reported following chronic stress and is associated with depressive-like behaviors in male rats (Li et al., 2011). Further, blocking NMDA or AMPA receptors in unstressed males recapitulates stress-induced deficits in behavioral flexibility (Jett et al., 2017). Thus, reduced glutamate neurotransmission likely plays an important role in stress-induced deficits in behaviors that are mPFC-mediated. Notably, the effect of stress on glutamatergic neurotransmission appears to be more pronounced in males than females (Wei et al., 2014).

Recent studies have also shown marked changes in the prefrontal GABAergic network following chronic stress. For example, unlike pyramidal neurons in mPFC of male rats, dendritic outgrowth occurs in GABAergic interneurons following chronic stress (Gilabert-Juan et al., 2013), although a reduction in the number of GABAergic cells has also been reported (Czéh et al., 2018). Only recently have potential sex differences in the effects of chronic stress on GABAergic neurotransmission been investigated. For instance, female mice exposed to 4 weeks of chronic unpredictable mild stress have an increase in the expression of parvalbumin mRNA in mPFC, and c-Fos expression is increased in parvalbumin-expressing cells (Shepard et al., 2016). Further, chronically stressed female mice have greater glutamatergic transmission onto parvalbumin-expressing neurons in mPFC (Shepard and Coutellier, 2018). These findings

suggest that the inhibitory tone in mPFC may be enhanced in chronically stressed female mice. Notably, these changes in GABAergic neurotransmission are largely absent in male mice following chronic stress (Shepard et al., 2016; Shepard and Coutellier, 2018). Together, these studies suggest that chronic stress-induced changes in the prefrontal GABAergic system are more pronounced in females, while changes in glutamatergic neurotransmission are more pronounced in males.

It is currently unknown if these changes in glutamatergic and GABAergic neurotransmission in mPFC are persistent, if they abate after a post-stress rest period, or if there are post-stress changes distinct from those that occur immediately following chronic stress. I have shown that dendritic reorganization of pyramidal neurons, the primary glutamatergic neurons in the cerebral cortex, is sex-specific during the post-chronic stress period (Chapter 2; Moench and Wellman, 2017). Additionally, there are persistent sex differences in neuronal activation in mPFC following a novel acute stressor in chronically stressed rats (Chapter 3; Moench et al., 2019), and females, unlike males, are markedly impaired on an extradimensional set shifting task after 2-hit stress (Chapter 4). These findings suggest that there may be persistent sex-specific changes in glutamatergic and/or GABAergic neurotransmission in mPFC during the post-chronic stress rest period.

Here I test this hypothesis by quantifying the expression of a subset of genes related to glutamatergic and GABAergic signaling immediately following chronic stress, following a post-stress rest period, and following two-hit stress in prelimbic cortex (PL) of male and female rats. For glutamate-related genes, four receptor subunits will be examined: NR1, NR2A, NR2B, and GluR1. NR1 is the obligate NMDA receptor subunit, while NR2A and NR2B are the next two most abundant NMDA receptor subunits, the latter of which is thought to play an important role in synaptic plasticity (reviewed in Cull-Candy and Leszkiewicz, 2004). Likewise, GluR1 is an AMPA receptor subunit that has received considerable attention for its role in synaptic plasticity (Lee et al., 2003). Together, the relative abundance of these subunits likely plays an important role in the

excitatory tone of circuits crucial for learning and memory (Goldman-Rakic, 1995). The expression of three GABA-related genes will also be measured: Gad67, somatostatin (SST), and parvalbumin (PV). Gad67 is one of two primary enzymes that convert glutamate to GABA in the central nervous system (Martin and Rimvall, 1993). The most abundant class of GABAergic interneurons in the prefrontal cortex are those that contain PV. These cells are thought to play a major role in regulating the spike timing of nearby pyramidal neurons (reviewed in Ferguson and Gao, 2018). Neurons that contain the neuropeptide SST also constitute a large proportion of inhibitory neurons in prefrontal cortex, and largely target synaptic inputs onto the distal tufts of pyramidal neurons (Rudy et al., 2011). Importantly, both PV and SST neurons have recently gained attention for playing important, yet distinct, roles in modulating working memory (Kim et al., 2016). Altogether, sex-specific changes in the relative abundance of these genes following chronic and two-hit stress could contribute to sex differences in stress-induced behavioral changes by altering the excitation/inhibition balance in medial prefrontal cortex.

Materials and Methods

Subjects and Stressors

Male and female Sprague Dawley rats (approximately 10 weeks of age at start; Envigo, Indianapolis, IN) were group-housed (3/cage) in standard laboratory cages (48 cm × 20 cm × 26 cm), with ambient temperature 23-25 °C, free access to food and water, and a 12:12 light/dark cycle (lights on at 0800 h). All procedures were conducted between 8:00 am and 6:00 pm, were in accordance with NIH Guidelines, and were approved by Indiana University's IACUC.

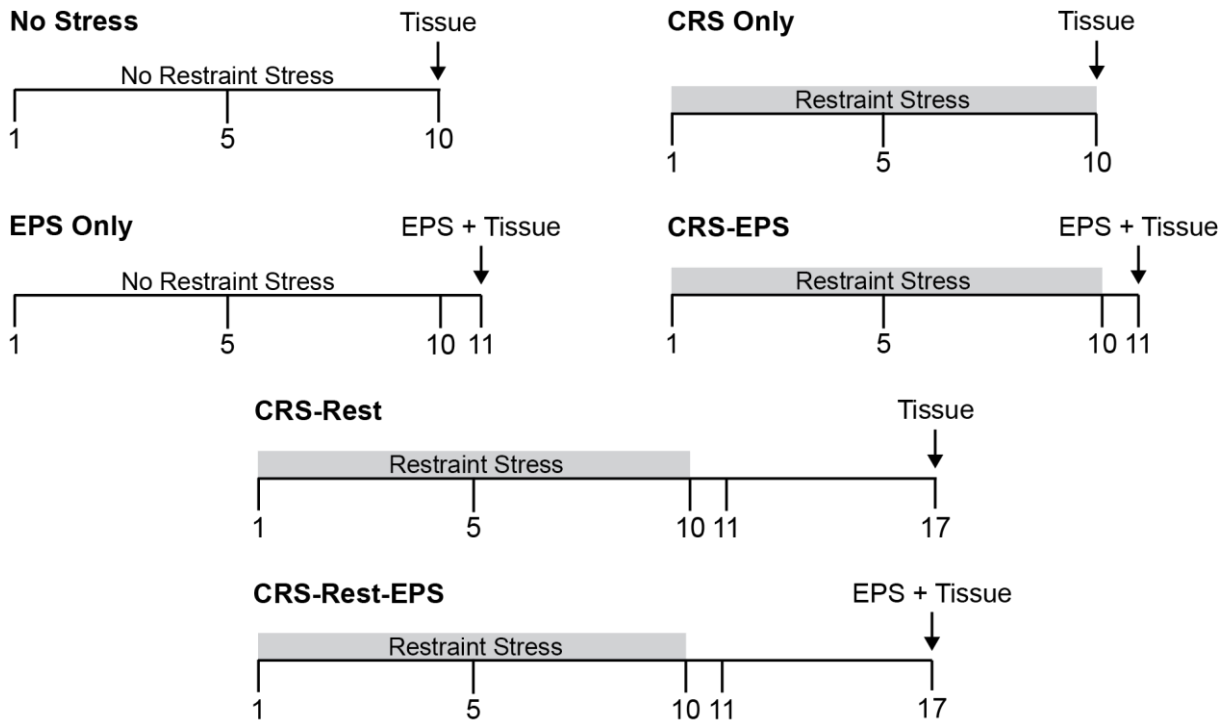


Figure 5.1. Experimental design and timeline. Male and female rats were either left undisturbed or exposed to CRS for 10 days. Rats either remained unstressed (No Stress) or were exposed to EPS (EPS Only). CRS rats were either 1) euthanized on the final day of restraint (CRS Only); 2) exposed to EPS on the day following CRS prior to euthanasia (CRS-EPS); 3) given a 7 day rest period following CRS (CRS-Rest); or 4) exposed to EPS 7 days following the cessation of CRS (CRS-Rest-EPS).

Chronic stress consisted of daily restraint (CRS; 3 h/day, 10 d). Rats were weighed daily throughout the stress procedure. Immediately after weighing, unstressed rats were returned to their home cages and left undisturbed in a separate room. Chronically stressed rats were placed in semi-cylindrical restrainers (male, 16 cm length × 6.5 cm width × 5 cm height; female, 15 cm length × 6 cm width × 4.5 cm height, modified so the tail piece locks into place; Braintree Scientific, Braintree, MA) in their home cages, with the time of restraint unpredictably varied over the light cycle. This manipulation produces significant increases in plasma corticosterone levels (Cook and Wellman, 2004). Elevated platform stress (EPS) was used as an acute stressors and consisted

of placing each rat individually on a small platform (12 cm × 12 cm) elevated 90 cm off the ground for 30 min in a brightly lit room as previously described (Xu et al., 1997; Maroun and Richter-Levin, 2003; Maroun et al., 2013). Rats were assigned to one of six stress conditions (see Fig. 5.1 for experimental design): No Stress (n = 9/sex); EPS Only (n = 9/sex); chronic stress only (CRS Only; n = 8 male, 9 female); chronic stress followed by EPS 1 day later (CRS-EPS; n = 8 male; 9 female); chronic stress followed by a 7 day rest period (CRS-Rest; n = 9 male, 7 female); or chronic stress followed by EPS 7 days later (CRS-Rest-EPS; n = 9/sex). Body weight change (Day 10 – Day 1) was analyzed using a two-way ANOVA (stress x sex) followed by Fisher's protected LSD *post-hoc* comparisons to verify the chronic stress manipulation.

Gene Expression Analysis

Tissue Collection and RNA Isolation. Animals were overdosed with urethane and rapidly decapitated. Brains were extracted, snap-frozen on dry ice, and stored at -80 ºC. Slices through prefrontal cortex (1 mm) were obtained using a precision brain slicer (Braintree Scientific Inc., Braintree, MA) that was equilibrated to -20 ºC. Prelimbic cortex was identified using gross anatomical landmarks (Paxinos and Watson, 1998) and micropunch samples (1.5 mm diameter) were obtained from both hemispheres (Fig. 5.2). Total RNA was isolated using the Maxwell RSC simplyRNA Tissue Kit (Promega, Madison, WI) per the manufacturer's recommended protocol. RNA concentration was determined using the Take3 Micro-Volume Plate and BioTek spectrophotometer (BioTek, Winooksi, VT).

cDNA synthesis. Aliquots of RNA samples (11 µL) were DNAse treated for 30 min at 37 ºC followed by 65 ºC for 10 min. Samples were then chilled to 4 ºC prior to being incubated with oligo-dT primers (1.5 µL, 5 µM, Invitrogen) and dNPTs (0.5 µL, 25 mM, Invitrogen) for 10 min at 65 ºC. Reverse transcription into first strand cDNA then proceeded via the addition of a mixture of SuperScript III Reverse Transcriptase (1 µL, Invitrogen), an RNAse inhibitor (1 µL, RNAseOUT,

Invitrogen), First Strand Buffer (4 µL, 5X, Invitrogen), and DTT buffer (1 µL, 0.1M, Invitrogen). Samples were then incubated at 42 ºC for 50 min followed by enzyme denaturation and reaction termination at 70 ºC for 15 min. cDNA samples were stored at -20 ºC.

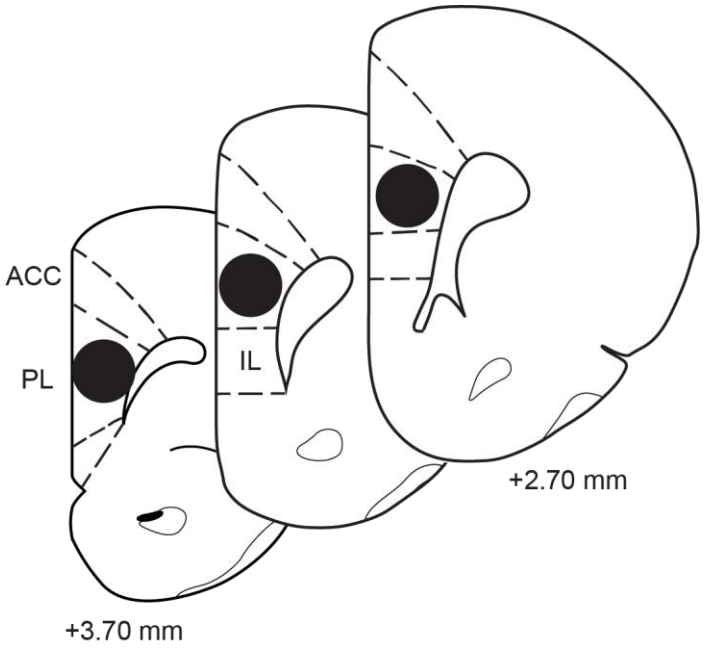


Figure 5.2. Schematic diagram depicting location of micropunch samples from prelimbic (PL) cortex. Samples were obtained using gross morphological criteria (Paxinos and Watson, 1998). For simplicity, only one hemisphere is shown.

Quantitative PCR. Primers to measure the expression of genes of interest, as well as the reference gene glyceraldehyde-6-phosphate dehydrogenase (GAPDH), were selected from previous studies and optimized for the current study (Table 5.1). Primers were obtained from Eurofins Genomics (Eurofins MWG Operon LLC, Huntsville, AL). For each sample, triplicate reactions were performed in 384-well plates at a volume of 10 µL. Reactions consisted of 3 µL cDNA, 5 µL PerfeCta SYBR Green SuperMix (Quanta BioSciences, Gaithersburg, MD), 1.2 µL primer, and 0.8 µL UltraPure water. Formation of PCR product was measured in real time using the Roche LightCycler 480 System (Roche Diagnostics, Indianapolis, IN) with cycling conditions as previously described (Bollinger et al., 2016). PCR cycling began with a pre-incubation phase (10 min, 95 ºC) followed by 45 cycles of denaturation (15 s, 95 ºC), annealing (40 s, 60 ºC), and product extension (10 s, 72 ºC). SYBR green I fluorescence was captured at 72 ºC. Primer specificity and primer dimer formation were assessed post-cycling using melt curves.

The relative abundance of mRNA was quantified using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). Mean C_T values were calculated across reaction triplicates for each sample. The mean C_T of the internal reference gene was subtracted from this value, which was then normalized via subtraction of the mean C_T of the internal reference gene from the respective gene of interest of the sample measured across all plates. To compare basal sex difference in the expression of genes of interest, data from No Stress female rats were expressed relative to No Stress male rats. These data were compared using independent samples t-tests. Following this initial comparison, data for all stress conditions were expressed relative to same-sex No Stress controls. These data were compared using one-way ANOVAs. To limit the number of extraneous comparisons, significant ANOVAs were followed by planned comparisons that 1) compared all groups to the No Stress condition, 2) compared groups that were exposed to only one stressor, 3) compared all groups exposed to EPS, and 4) compared all groups exposed to CRS.

Table 5.1. Primer specifications for qPCR.

Gene	Function	Primer Sequence (5' – 3')	Reference
NR1	Obligate NMDA receptor subunit	F – TGGTAGAGCAGAGCCCGACCC R – CCCCGGTGCTCGTGTCTTTGG	Lopes et al. (2013)
NR2A	NMDA receptor subunit	F – AGCCCCCTTCGTCATCGT R – GACAGGGCACCGTGTTCCT	Pershing et al. (2016)
NR2B	NMDA receptor subunit	F – CCCAACATGCTCTCTCCCTTAA R – CAGCTAGTCGGCTCTCTTGGTT	Lindenbach et al. (2015)
GluR1	AMPA receptor subunit	F – ATGCTGACCTCCTTCTGTGG R – TCCTGTAGTTCCGGGCGTAG	Sadri-Vakili et al. (2010)
Gad67	GABA synthesis enzyme	F – GCTGGAAGGCATGGAAGGTTTTA R – ACGGGTGCAATTTCATATGTGAACATA	Jaenisch et al. (2014)
PV	Calcium-binding protein	F – AGCCTTTACTGCTGCAGACTCCTT R – AGCTCATCCTCCTCAATGAAGCCA	Bastian et al. (2014)
SST	Neuropeptide	F – GCCACCGGGAAACAGGAACTGG R – GGGTGCCATGGCTGGGTTCG	Hou and Yu (2013)
GAPDH	Glycolysis	F – ACCACAGTCCATGCCATCACTG R – GATGACCTTGCCCACAGCCTT	Bollinger et al. (2016)

Results

Body Weight Analysis

Male rats gained significantly more weight than female rats (Fig. 5.3; main effect of sex, $F_{(1, 92)} = 6.64$, $p = 0.01$) and stress altered weight change in both males and females (main effect of stress, $F_{(5, 92)} = 38.45$, $p < 0.001$), although this effect was more pronounced in males (sex × stress interaction, $F_{(5, 92)} = 4.36$, $p = 0.001$). In males, weight gain did not differ between No Stress and EPS Only rats. In contrast, male rats exposed to chronic stress gained significantly less weight from Day 1 through Day 10 than No Stress male rats (CRS Only, $p < 0.001$; CRS-EPS, $p < 0.001$; CRS-Rest, $p < 0.001$; CRS-Rest-EPS, $p < 0.001$). Similarly, in female rats, weight change did not differ between No Stress and EPS Only rats, whereas in chronically stressed females, weight change was significantly reduced relative to No Stress female rats (CRS Only, $p < 0.001$; CRS-EPS, $p < 0.001$; CRS-Rest, $p < 0.001$; CRS-Rest-EPS, $p = 0.004$).

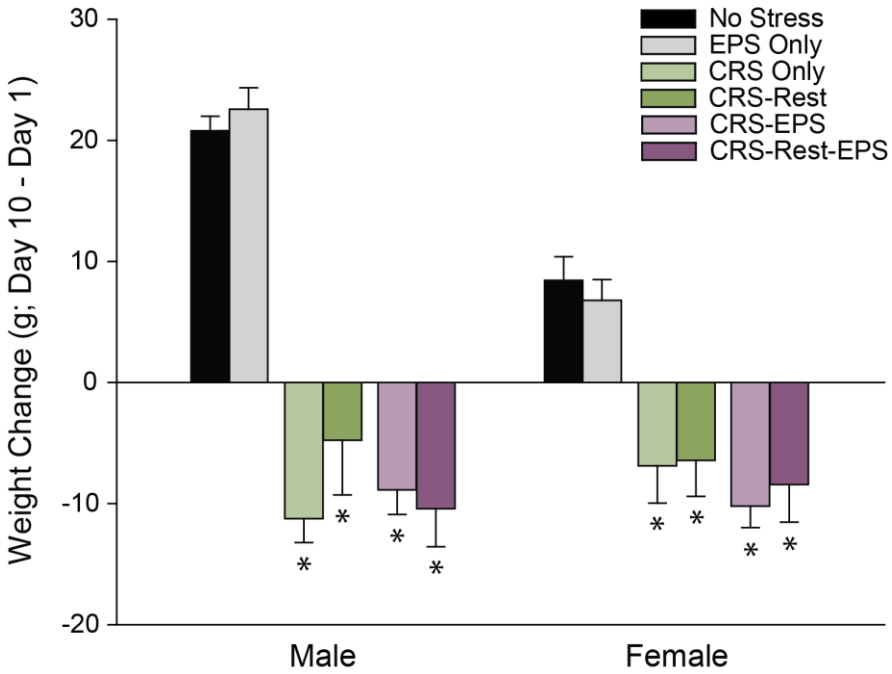


Figure 5.3. Chronic stress attenuates weight gain in both male and female rats. Error bars represent SEM. * $p < 0.05$ compared to No Stress rats of same sex.

Basal Sex Differences in Gene Expression

The relative expression of NR1 was significantly greater in No Stress female rats compared to No Stress male rats (Fig. 5.4; $t_{(15)} = -2.81$, $p = 0.01$). No other basal sex differences in relative gene expression were significant (t's ≤ 1.32, all n.s.).

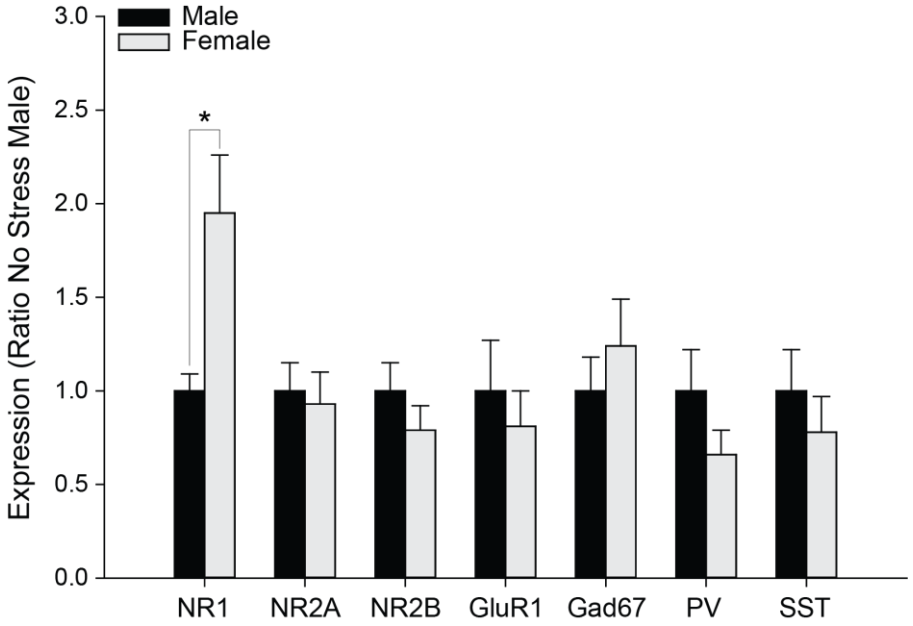


Figure 5.4. Basal sex differences in gene expression. Relative NR1 expression is higher in No Stress females than males. Error bars represent SEM. * $p < 0.05$ compared to No Stress rats of same sex.

Stress Effects on Glutamatergic Gene Expression in Males

The relative expression of NR1 differed significantly as a result of stress in male rats (Fig. 5.5 A; $F_{(5, 43)} = 7.18$, $p < 0.001$). Planned comparisons revealed that the expression of NR1 was greater in EPS Only ($F_{(1, 15)} = 18.69$, $p = 0.001$), CRS Only ($F_{(1, 14)} = 5.94$, $p = 0.03$), CRS-Rest $F_{(1, 14)} = 18.69$, $p < 0.001$), and CRS-Rest-EPS ($F_{(1, 14)} = 10.79$, $p = 0.005$) males compared to No Stress males, while expression in CRS-EPS males did not differ from controls ($F_{(1, 14)} = 1.82$, n.s.). The EPS-induced increase in the expression of NR1 was prevented by CRS, but only if a rest period was not present (EPS Only v CRS-EPS, $F_{(1, 15)} = 4.40$, $p = 0.05$; EPS Only v CRS-Rest-EPS, $F_{(1, 15)} = 0.63$, n.s.). The CRS-induced increase in NR1 expression was enhanced following

a rest period (CRS Only v CRS-Rest, $F_{(1, 14)} = 14.44$, $p = 0.002$), but this enhancement was blunted following exposure to EPS (CRS-Rest v CRS-Rest-EPS, $F_{(1, 14)} = 8.15$, $p = 0.01$). Despite this, NR1 expression was greater in CRS-Rest-EPS males compared to CRS-EPS males ($F_{(1, 14)} = 4.51$, $p = 0.05$). No other planned comparisons reached significance (F's ≤ 1.25, all n.s.). The relative expression of NR2A and NR2B (Table 5.2) did not differ significantly as a result of stress in male rats (NR2A, $F_{(5, 45)} = 1.78$, n.s.; NR2B, $F_{(5, 45)} = 1.42$, n.s.).

Stress significantly altered the relative expression of GluR1 in males (Fig. 5. 5 C; $F_{(5, 43)} = 2.87$, $p = 0.03$). Planned comparisons revealed that EPS increased expression of GluR1 (EPS Only v No Stress, $F_{(1, 16)} = 5.12$, $p = 0.04$). GluR1 expression was not alter by CRS alone but was increased in CRS males following a rest period (No Stress v CRS Only, $F_{(1, 15)} = 0.52$, n.s.; No Stress v CRS-Rest, $F_{(1, 16)} = 4.46$, $p = 0.05$). The EPS-induced increase in the expression of NR1 was prevented by CRS, but only if a rest period was not present (EPS Only v CRS-EPS, $F_{(1, 15)} = 13.93$, $p = 0.002$; EPS Only v CRS-Rest-EPS, $F_{(1, 15)} = 0.13$, n.s.), resulting in a significant difference between CRS-EPS and CRS-Rest-EPS males ($F_{(1, 14)} = 7.05$, $p = 0.02$). No other planned comparisons reached significance (F's ≤ 2.63, all n.s.).

Stress Effects on Glutamatergic Gene Expression in Females

In females, stress significantly altered the relative expression of NR1 (Fig. 5.5 B; $F_{(5, 43)} = 3.80$, $p = 0.006$). Planned comparisons revealed that whereas EPS and CRS alone did not alter NR1 expression (No Stress v EPS Only, $F_{(1, 15)} = 1.45$; No Stress v CRS Only, $F_{(1, 15)} = 0.03$, n.s.), the combination of the two did, but only when a rest period was present (No Stress v CRS-EPS, $F_{(1, 16)} = 0.002$, n.s.; No Stress v CRS-Rest-EPS, $F_{(1, 15)} = 7.56$, $p = 0.02$; CRS-EPS v CRS-Rest-EPS, $F_{(1, 15)} = 8.05$, $p = 0.01$). Further, NR1 expression was greater in CRS-Rest-EPS females compared to CRS Only ($F_{(1, 14)} = 7.76$, $p = 0.02$) and CRS-Rest ($F_{(1, 13)} = 14.38$, $p = 0.002$) females. No other planned comparisons reached significance (F's ≤ 2.28, all n.s.). The relative expression

of NR2A and NR2B (Table 5.2) did not differ significantly as a result of stress in female rats (NR2A, $F_{(5, 43)} = 1.36$, n.s.; NR2B, $F_{(5, 44)} = 2.01$, n.s.).

The relative expression of GluR1 was significantly altered by stress in females (Fig. 5.5 D; $F_{(5, 45)} = 2.36$, $p = 0.05$). Planned comparisons revealed that GluR1 expression was increased in CRS females either after a rest period (No Stress v CRS-Rest, $F_{(1, 14)} = 4.67$, $p = 0.05$) or after exposure to EPS without a rest period (No Stress v CRS-EPS, $F_{(1, 16)} = 5.13$, $p = 0.04$). GluR1 expression was also greater in CRS-EPS females compared to EPS only females ($F_{(1, 15)} = 8.79$, $p = 0.01$). No other planned comparisons reached significance (F's ≤ 3.34, all n.s.).

Stress Effects on GABAergic Gene Expression in Males

The relative expression of Gad67 differed significantly as a result of stress in males (Fig 5.6 A; $F_{(5, 44)} = 2.09$, $p = 0.005$). Expression of Gad67 was not altered by CRS alone, but was increased in CRS males following a rest period (No Stress v CRS Only, $F_{(1, 15)} = 0.31$, n.s.; No Stress v CRS-Rest, $F_{(1, 15)} = 6.90$, $p = 0.02$; CRS Only v CRS-Rest, $F_{(1, 14)} = 8.14$, $p = 0.01$). Further, Gad67 expression was greater in CRS-Rest-EPS males compared to CRS Only males ($F_{(1, 15)} = 5.59$, $p = 0.03$). No other planned comparisons reached significance (F's ≤ 3.63, all n.s.).

The relative expression of PV in males also differed significantly as a result of stress (Fig. 5.6 C; $F_{(5, 45)} = 5.73$, $p < 0.001$). Planned comparisons revealed that while EPS (No Stress v EPS Only, $F_{(1, 16)} = 3.55$, n.s.) and CRS (No Stress v EPS Only, $F_{(1, 14)} = 0.01$, n.s.) alone did not alter PV expression, both CRS-Rest and CRS-Rest-EPS males had greater PV expression than No Stress males (CRS-Rest, $F_{(1, 16)} = 15.23$, $p = 0.001$; CRS-Rest-EPS, $F_{(1, 16)} = 5.73$, $p = 0.03$), and CRS Only males (CRS-Rest, $F_{(1, 14)} = 12.76$, $p = 0.003$; CRS-Rest-EPS, $F_{(1, 14)} = 4.81$, $p = 0.05$). Further, PV expression was significantly reduced in CRS-EPS males compared to EPS Only males ($F_{(1, 15)} = 6.01$, $p = 0.03$) and CRS-Rest-EPS males ($F_{(1, 15)} = 7.79$, $p = 0.01$). No other planned comparisons reached significance (F's ≤ 3.14, all n.s.). The relative expression of SST in males did not differ across stress conditions (Table 5.2; $F_{(5, 44)} = 1.25$, n.s.).

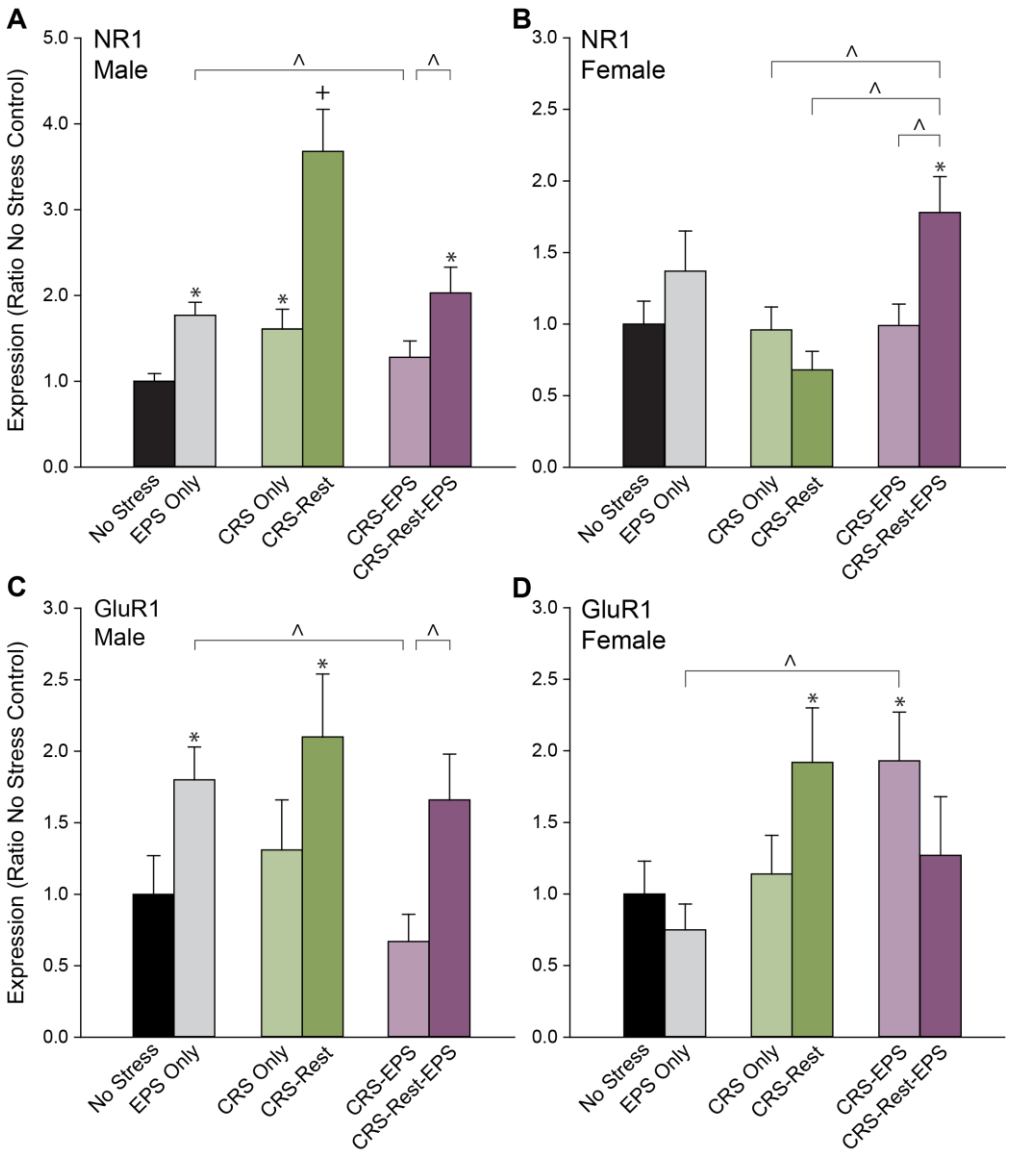


Figure 5.5. Expression of NR1 and GuR1 in males and females. (A) In males, both EPS and CRS alone increased NR1 expression. This increase following CRS was enhanced following a rest period. The combination of EPS and CRS also resulted in increased NR1, but only when a rest period was present. (B) In females, the combination of CRS and EPS increased the expression of NR1 only when a rest period was present. (C) EPS alone increased GluR1 expression in males, but this increase was prevented when EPS was immediately preceded by CRS. GluR1 expression was not altered by CRS alone, but was greater in CRS-Rest males. (D) GluR1 expression was increased in CRS females after a rest period or after exposure to EPS without a rest period. Error bars represent SEM. * $p < 0.05$ compared to No Stress; $^{+}$ $p < 0.05$ compared to all other groups; $^{\wedge}p < 0.05$.

Stress Effects on GABAergic Gene Expression in Females

In female rats, the relative expression of Gad67 (Fig. 5.6 B) and SST (Table 5.2) did not differ significantly as a result of stress (Gad67, $F_{(5, 44)} = 2.00$, n.s.; SST, $F_{(5, 46)} = 1.19$, n.s.). In contrast, the relative expression of PV was significantly altered by stress in females Fig. 5.6 D; $F_{(5, 46)} = 2.38$, $p = 0.05$). Planned comparisons revealed that CRS significantly increased the expression of PV in females ($F_{(1, 16)} = 7.13$, $p = 0.02$), and this increase was still present following a rest period $F_{(1, 14)} = 6.95$, $p = 0.02$). In contrast, PV expression was reduced following EPS in CRS females after a post-CRS rest period $F_{(1, 16)} = 4.41$, $p = 0.05$). No other planned comparisons reached significance (F's ≤ 3.09, all n.s.).

Table 5.2. Stress effects on NR2A, NR2B, and SST gene expression of males and females.

Sex	Stress	NR2A ($2^{-\Delta\Delta CT}$ ± SEM)	NR2B ($2^{-\Delta\Delta CT}$ ± SEM)	SST ($2^{-\Delta\Delta CT}$ ± SEM)
Male	No Stress	1.00 ± 0.15	1.00 ± 0.15	1.00 ± 0.22
	EPS Only	1.97 ± 0.37	1.14 ± 0.18	1.32 ± 0.06
	CRS Only	1.14 ± 0.27	1.36 ± 0.29	1.31 ± 0.23
	CRS-Rest	1.56 ± 0.23	0.97 ± 0.15	1.00 ± 0.17
	CRS-EPS	1.22 ± 0.39	0.68 ± 0.12	0.78 ± 0.15
	CRS-Rest-EPS	1.83 ± 0.33	1.07 ± 0.17	1.17 ± 0.21
Female	No Stress	1.00 ± 0.18	1.00 ± 0.17	1.00 ± 0.25
	EPS Only	1.42 ± 0.24	1.36 ± 0.23	1.17 ± 0.20
	CRS Only	1.08 ± 0.17	0.92 ± 0.21	1.13 ± 0.19
	CRS-Rest	1.78 ± 0.35	1.62 ± 0.31	1.40 ± 0.24
	CRS-EPS	1.27 ± 0.17	1.20 ± 0.26	1.09 ± 0.18
	CRS-Rest-EPS	1.17 ± 0.27	0.69 ± 0.16	0.70 ± 0.17

Note: $2^{-\Delta\Delta CT}$ values for each stress condition are expressed relative to No Stress males and females.

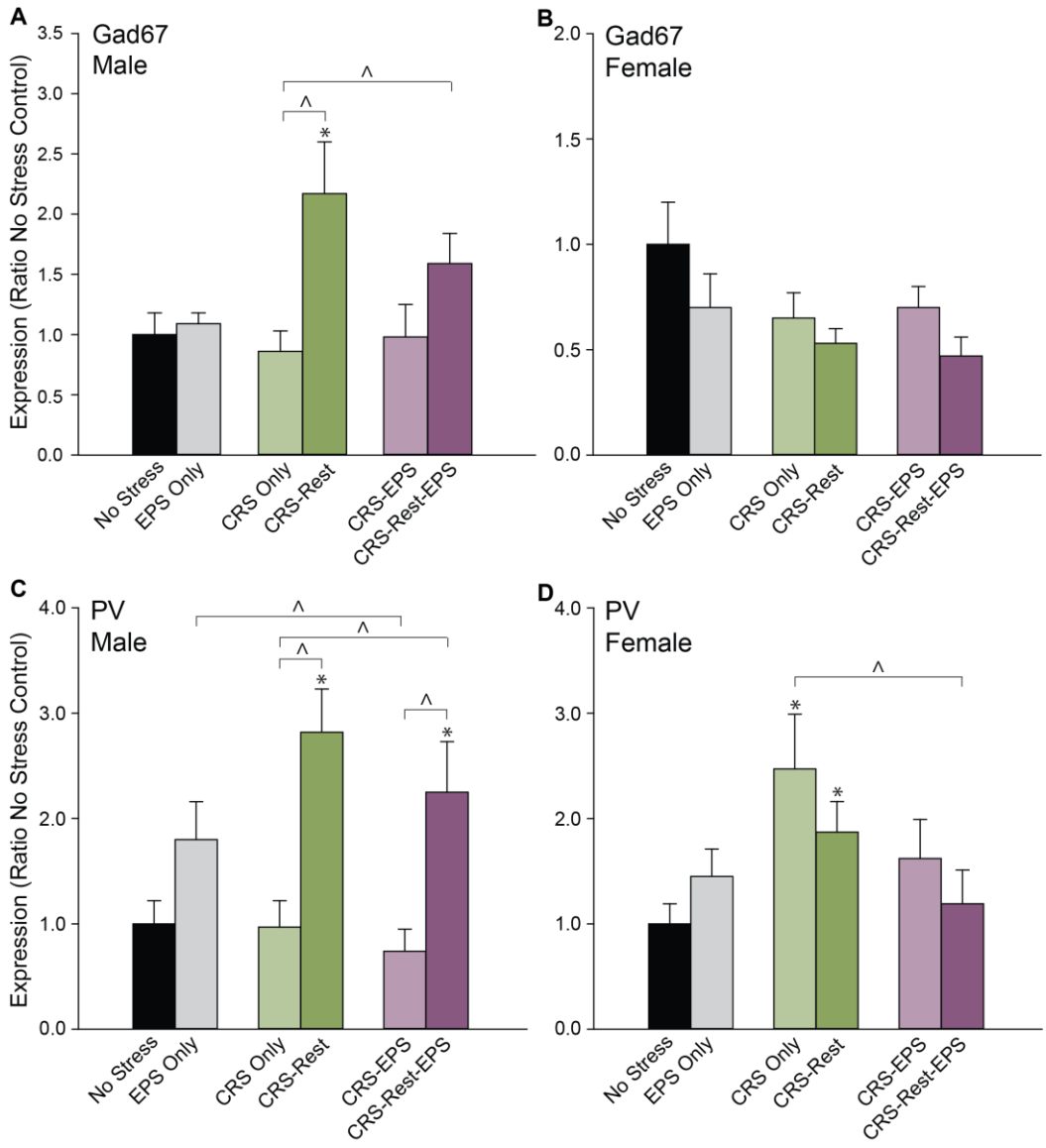


Figure 5.6. Expression of Gad67 and PV in males and females. (A) Gad67 expression was not altered by EPS or CRS alone, but was increased in CRS males following a rest period. This increase following CRS and rest period was also present following EPS. (B) Gad67 expression was not altered by stress in females. (C) PV expression was not altered by EPS or CRS, but was increased in CRS-Rest males. PV expression was also increased in males that were exposed to both EPS and CRS, but this was only the case if a rest period separated the two. (D) CRS significantly increased the expression of PV in females, and this increase was still present following a rest period. In contrast, PV expression was reduced following EPS in CRS females after a post-CRS rest period. Error bars represent SEM. * $p < 0.05$ compared to No Stress; ^ $p < 0.05$.

Discussion

Data from this chapter indicate that acute, chronic, and two-hit stress alter the expression of NR1 (Fig. 5.7), GluR1 (Fig. 5.8), Gad67 (Fig. 5.9), and PV (Fig. 5.10) genes in a sex-specific manner.

Sex differences in the effects of stress on the expression of NR1 and GluR1 mRNA.

Both acute and chronic stress are known to affect glutamatergic neurotransmission in mPFC of males through postsynaptic NMDA receptor modulation (reviewed in Yuen et al., 2017). Following acute stress, the surface expression of NR1 subunits is increased in mPFC of male rats (Yuen et al., 2009). The data presented here suggest this increase in NR1 also occurs at the level of mRNA expression in males. In contrast to the effects of acute stress, chronic stress has been shown to downregulate the expression of NR1 protein expression in males (Yuen et al., 2012; Wei et al., 2014), although others have reported no change in NR1 mRNA expression 48 hours after the cessation of stress (Shepard and Coutellier, 2018). It is possible that the increase in NR1 mRNA found here reflects an acute stress-induced increase in expression given that tissue was taken on the final day of stress. Further, it is now well understood that post-translational modifications of mRNA result in poor correlations between gene expression changes and protein levels following a number of experimental manipulations (Vogel and Marcotte, 2012). This could explain the discrepancy in mRNA expression found here and the reduction in the expression of NR1 subunits found in previous studies (Yuen et al., 2012; Wei et al., 2014), and suggests that there might be post-translational modifications that take place to result in an overall downregulation of protein expression.

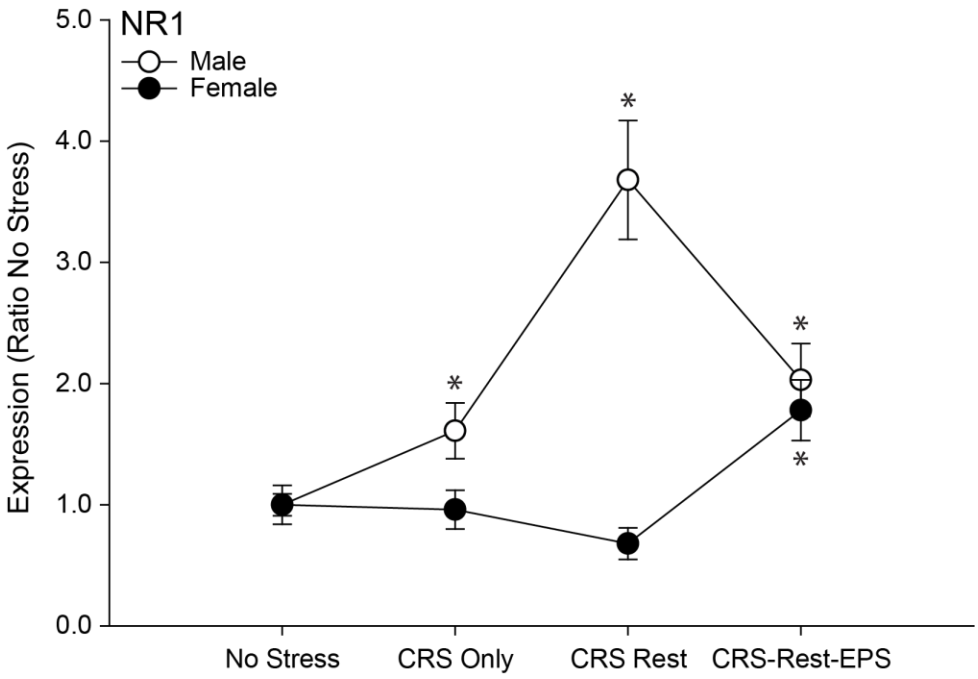


Figure 5.7. Longitudinal depiction of chronic and two-hit stress effects on NR1 mRNA expression in males and females. In males, NR1 expression was increased following chronic stress, a post-chronic stress rest period, and following exposure to a novel stress challenge. In female rats, NR1 expression was only increased following two-hit stress. Error bars represent SEM. * $p < 0.05$ compared to No Stress.

Despite these discrepancies, the data presented here clearly demonstrate that the effects of acute and chronic stress result in different patterns of NR1 mRNA expression between males and females. In the case of both acute and chronic stress, the expression of NR1 in females was unchanged. Although this is the first study to examine acute stress effects on NR1 expression, the lack of change following chronic stress is consistent with a lack of change in surface protein in mPFC that has been previously reported following chronic restraint stress (Wei et al., 2014) and mRNA expression following chronic unpredictable mild stress (Shepard and Coutellier, 2018).

The most novel, and perhaps most notable, findings are the sex differences that persist and/or emerge in NR1 expression following the cessation of chronic stress, and following the combination of acute and chronic stress. In chronically stressed males, the expression of NR1

was even greater following a rest period than that found immediately after chronic stress. However, this increase was not present following an acute stress challenge. In contrast, when no rest period was given between chronic and acute stress exposure, NR1 expression was reduced compared to males exposed only to acute stress. One possible interpretation of this pattern is that while chronic stress leads to a reduction in NR1 expression following an immediate acute stress challenge, if a rest period is given, the typical pattern of acute stress-induced change in NR1 expression emerges. This is in striking contrast to the pattern observed in females, where no change in NR1 expression was found until chronically stressed rats were exposed to a novel stress challenge following a rest period. Thus, it appears that there may be a sort of 'priming' effect of chronic stress in females on the pattern of change in NR1 expression that emerges following acute stress that is not present following acute stress alone.

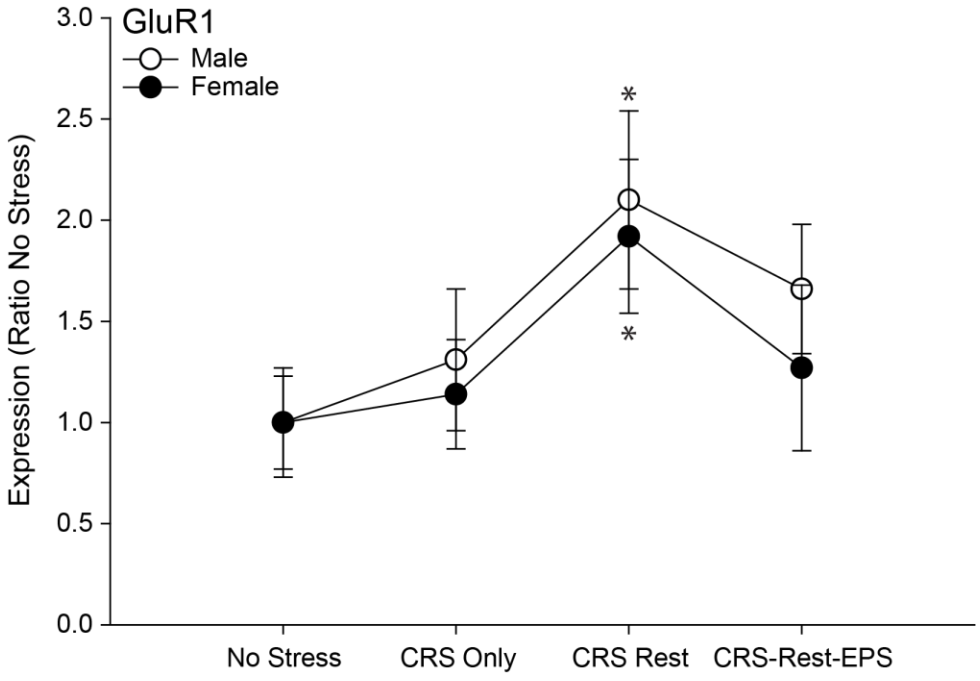


Figure 5.8. Longitudinal depiction of chronic and two-hit stress effects on GluR1 mRNA expression in males and females. In both males and females, GluR1 expression was increased only following a post-chronic stress rest period. Error bars represent SEM. * $p < 0.05$ compared to No Stress.

Like the expression of NR1, both acute and chronic stress can modulate the expression of AMPA receptor subunits, including GluR1 in mPFC of males. Also like NR1, the surface expression of GluR1 protein is increased following acute stress (Yuen et al., 2009), but decreased following chronic stress (Yuen et al., 2012; Wei et al., 2014) in males. In contrast, the expression of Glur1 protein is unchanged in females following chronic stress (Wei et al., 2014). Here, males had an increase in GluR1 mRNA following acute stress, but no change following chronic stress. In contrast, females had no change in GluR1 mRNA expression following acute or chronic stress. Notably, the combination of chronic and acute stress resulted in a downregulation in GluR1 expression in males, but an increase in expression in females. Further, in both males and females, GluR1 expression was elevated following a post-chronic stress rest period.

Given that greater levels of NR1 and GluR1 expression contribute to increases in NMDA- and AMPA receptor-mediated synaptic currents (Yuen et al., 2017), it is possible that the sex-specific changes in the expression of these receptor subunits could contribute to functional differences in prelimbic cortex in males and females at these different post-chronic stress timepoints.

Sex differences in the effects of stress on the expression of Gad67 and PV mRNA.

Several recent studies have revealed that stress can have profound effects on the prefrontal GABAergic system, although the findings from these studies are mixed. In males, 14 days of both unpredictable chronic mild stress (Shepard et al., 2016) and chronic social subordination (Makinson et al., 2015) result in increased Gad67 mRNA expression in mPFC. In contrast, 36 days of chronic unpredictable stress leads to a reduction in Gad67 protein expression in males (Banasr et al., 2017). In females, no change in Gad67 mRNA expression has been reported after chronic mild stress (Shepard et al., 2016). The data presented here also show no change in Gad67 in females. In contrast, while chronic stress alone did not alter Gad67 expression in males, an increase was found following a post-chronic stress rest period. This increase was

partially prevented following an acute stress challenge. Given that Gad67 is a primary enzyme in the production of GABA, the increase in Gad67 expression following the cessation of chronic stress suggests that there is likely greater inhibitory tone in mPFC of chronically stressed male rats during this time.

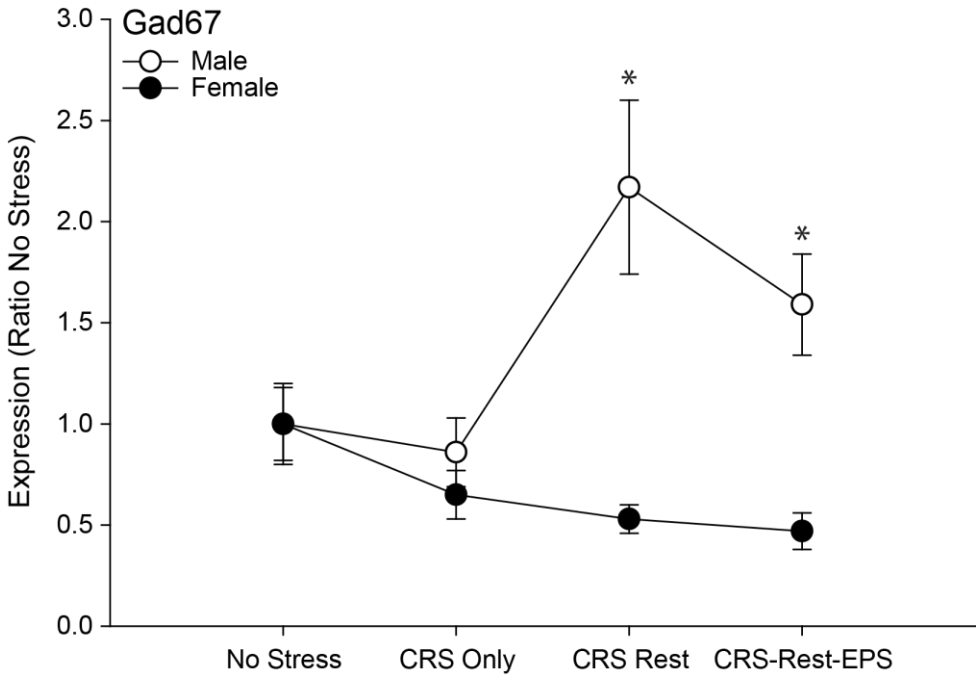


Figure 5.9. Longitudinal depiction of chronic and two-hit stress effects on Gad67 mRNA expression in males and females. In males, Gad67 expression was increased following a post-chronic stress rest period and following exposure to a novel stress challenge. In female rats, Gad67 expression was unaltered by both chronic and two-hit stress. Error bars represent SEM. * $p < 0.05$ compared to No Stress.

Previous studies examining the expression of PV in response to stress have also yielded mixed findings. Both no change (Shepard et al., 2016; Czéh et al., 2018) and decreases (Todorović et al., 2019) in the number of PV-positive cells in mPFC of males have been found. A decrease in the total amount of PV protein has also been reported (Banasr et al., 2017). In contrast, in females, an increase in both PV-positive cells and mRNA occurs following chronic mild stress (Shepard et al., 2016; Shepard and Coutellier, 2018). Further, Shepard and Coutellier

(Shepard and Coutellier, 2018) determined that enhanced glutamatergic signaling could be driving increased PV expression in mPFC of females.

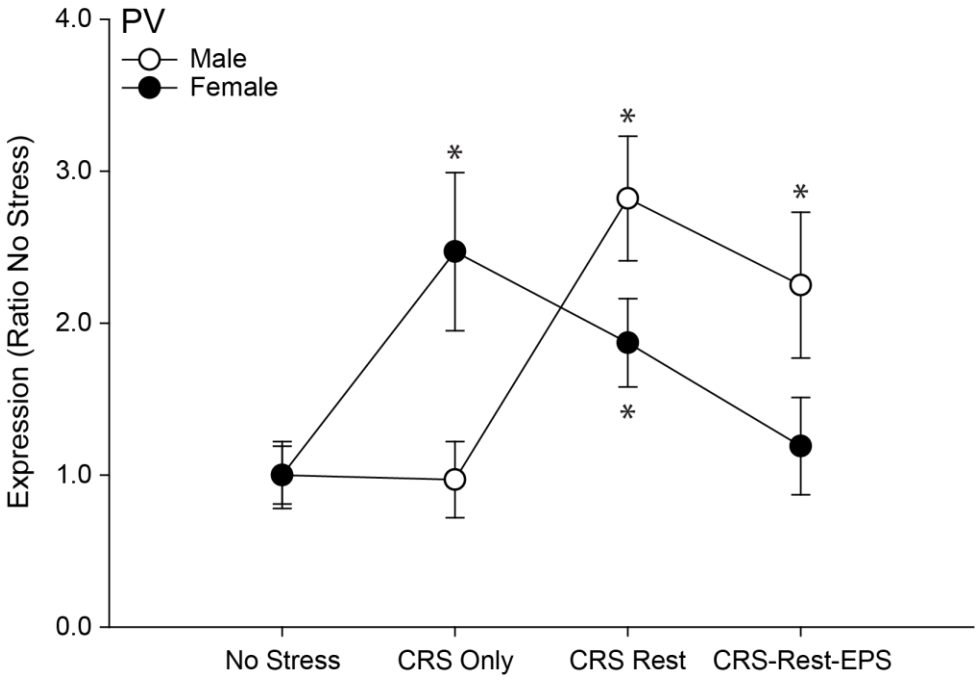


Figure 5.9. Longitudinal depiction of chronic and two-hit stress effects on PV mRNA expression in males and females. In males, PV expression was not altered following chronic stress, but was increased following a post-chronic stress rest period and following two-hit stress. In contrast, chronic stress increased PV expression in females. This increase was present following a post-chronic stress rest period, but not following two-hit stress. Error bars represent SEM. * $p < 0.05$ compared to No Stress.

Data from the current study show that neither acute nor chronic stress alter PV mRNA expression in males. However, PV expression was increased in chronically stressed males following a rest period. Additionally, this increase was still present following exposure to a novel acute stressor. In contrast, chronic stress resulted in a significant and persistent increase in PV mRNA expression in females. Further, unlike in males, this increase was no longer present following a novel stress challenge. PV-expressing interneurons account for ~40% of cortical interneurons. These cells synapse on the somata and proximal dendrites of pyramidal neurons, and thus contribute to the balance of excitation and inhibition in cortical brain regions by

modulating the spike timing of excitatory neurons (reviewed in Ferguson and Gao, 2018). Given this, the data presented here suggest that chronically stressed males may have greater PV-mediated inhibition in mPFC that is sustained through a novel stress challenge. In contrast, whereas females also have an initial chronic stress-induced increase in PV that persists through a rest period, it is not sustained through a novel stress challenge. This pattern suggests that the balance of excitation and inhibition in mPFC of chronically stressed males and females is likely altered in a sex-specific pattern following exposure to a novel stress challenge. Again, this should result in very different functional changes in mPFC of males versus females.

Conclusions

Data from this chapter indicated that there are sex-specific changes in the gene expression of NR1, GluR1, Gad67, and PV in prelimbic cortex following acute, chronic, and two-hit stress. These changes likely contribute to the balance of excitation and inhibition in prelimbic cortex. Changes in glutamatergic and GABAergic neurotransmission immediately after the cessation of chronic stress have been linked to stress-induced behavioral changes (Wei et al., 2014; Shepard et al., 2016; Shepard and Coutellier, 2018). Thus, the changes in gene expression in the present study may contribute to sex-specific alterations in neuronal activation (Chapter 3; Moench et al., 2019) and extradimensional set-shifting (Chapter 4) during the post-stress period and in response to two-hit stress. Further, the changes in gene expression that were found in females following chronic stress, a post-chronic stress rest period, and in response to two-hit stress suggest that females are not 'resilient' to the effects of chronic stress. Instead, there are clear sex-specific changes following chronic stress, suggesting that there are differences in stress adaptation mechanisms between males and females that result in differential responses to future stressors.

Chapter 6:

General Discussion

In this dissertation, I demonstrated that there are marked sex differences in the structure and function of mPFC during the post-chronic stress period and in response to a novel stress challenge. In Chapter 2, I showed that chronic stress-induced dendritic remodeling in prelimbic cortex during the post-stress period is sex-specific. In males, dendritic retraction immediately following chronic stress is ameliorated following a 10-day rest period. Notably, chronically stressed male rats have dendritic *outgrowth* following a 7-day rest period compared to unstressed males. In contrast, dendritic remodeling in females during this time is minimal. In Chapter 3, I went on to show that chronically stressed males, but not females, have a persistent reduction in novel stress-induced neuronal activation in prelimbic cortex. This was also the case across a number of other corticolimbic brain regions, with the notable exceptions of the paraventricular nucleus of the hypothalamus and the basolateral amygdala. In these two regions, chronically stressed female rats have *enhanced* neuronal activation in response to a novel stress challenge. In Chapter 4, I showed that these different patterns of neuronal activation in response to "two-hit" stress might contribute to sex-specific behavioral changes in an attentional set-shifting task. In males, chronic stress reversibly disrupts extradimensional set-shifting. In contrast, chronic stress did not impact set-shifting in females. Instead, chronically stressed female rats had a deficit in extradimensional set-shifting only after "two-hit" stress, whereas behavior in males was unaffected by a novel stress challenge. Finally, in Chapter 5, I showed that there are sex-specific changes in the gene expression of NR1, GluR1, Gad67, and parvalbumin following acute, chronic, and two-hit stress. Altogether, these data suggest that chronic stress likely leads to the recruitment of sex-specific stress adaptation mechanisms that contribute to sex differences in the structure and function of rat prelimbic cortex during the post-chronic stress period.

Sex differences in the lasting effects of chronic stress on mPFC.

Chronic stress has well-documented effects on the structure and function of mPFC in male rodents. These include deficits in behaviors mediated by mPFC (Beck and Luine, 1999, 2002; Liston et al., 2006; Bondi et al., 2008; Nikiforuk and Popik, 2011, 2013, 2014; Wei et al., 2014; Jett et al., 2017), reductions in pyramidal neuron dendritic length and branching (Cook and Wellman, 2004; Radley et al., 2004; Radley et al., 2005; Garrett and Wellman, 2009; Goldwater et al., 2009; Moench and Wellman, 2017), and reduced glutamatergic neurotransmission (Yuen et al., 2012; Wei et al., 2014; Jett et al., 2017). A small but growing literature suggests that chronic stress does not have the same effect on mPFC in females. Unlike males, females tend not to have deficits in mPFC-mediated behaviors (Beck and Luine, 2002; Wei et al., 2014; Snyder et al., 2015), have minimal change in dendritic architecture or dendritic outgrowth (Garrett and Wellman, 2009; Moench and Wellman, 2017), and have little change in glutamatergic neurotransmission (Wei et al., 2014). Whether these changes abate following the cessation of chronic stress or persist through a post-stress rest period has been given considerably less attention.

Radley and colleagues (2005) first demonstrated that chronic stress-induced dendritic retraction in males is reversible following a 21 day rest period. In Chapter 2, I showed that this process of dendritic "recovery" can occur more quickly, as the length of apical dendrites in chronically stressed males were similar to that in unstressed males by just 10 days post-stress (Moench and Wellman, 2017). Surprisingly, following a 7 day post-stress rest period, the length of apical dendrites in chronically stressed males was *longer* than that of unstressed males. This pattern suggests that the process of dendritic "recovery" in prelimbic cortex of males is not a simple return to baseline, but instead is a dynamic process. Data from Chapter 5 indicate that there are post-chronic stress changes in gene expression in prelimbic cortex of males. Here, chronic stress increased the expression of the NMDA receptor subunit NR1. Not only did this increase persist through the post-stress rest period, but it was greater compared to both unstressed and chronically stressed males. A similar pattern was found in the expression of the

AMPA receptor subunit GluR1, and the GABAergic genes Gad67 and parvalbumin. In these cases, chronic stress alone did not result in gene expression changes. Instead, following a rest period, the expression of all three of these genes was increased compared to unstressed males, and in the case of the GABAergic genes, compared to chronically stressed males. Interestingly, despite these striking changes, following a post-chronic stress rest period, the deficit in extradimensional set-shifting induced by chronic stress is no longer present (Chapter 4). Together, these data provide strong evidence that molecular and cellular changes can occur during the post-stress rest period that do not represent a return to unstressed conditions, but nonetheless do not prevent, and perhaps could facilitate, the "recovery" of behavioral deficits.

Data from studies of the hippocampus support this notion. In males, chronic stress leads to dendritic retraction of pyramidal neurons in hippocampus (Watanabe et al., 1992; Magarinos and McEwen, 1995; Conrad et al., 1999) and disrupts spatial working memory tasks that are hippocampus-dependent (Luine et al., 1994; Sousa et al., 2000; Bowman et al., 2002; Hoffman et al., 2011; McFadden et al., 2011). Both are reversed after a post-stress rest period (Conrad et al., 1999; Sousa et al., 2000). Recent work by Gray and colleagues (2014) has shown that there are striking patterns of changes in gene expression that occur in the hippocampus following both chronic stress and a post-chronic stress rest period. RNA microarrays revealed that hundreds of genes were up- or downregulated immediately following chronic stress, as was also the case following a post-chronic stress rest period. Notably, very few genes overlapped between these two conditions. That is, genes that were upregulated immediately following chronic stress were not the same genes that were subsequently downregulated following a post-stress rest period, and vice versa, resulting in a striking difference in gene expression profiles between unstressed males and chronically stressed males given a rest period (Gray et al., 2014). Given that behavioral deficits induced by chronic stress are ameliorated following a rest period, this pattern of changes in gene expression suggests that there are widespread changes in the post-stress physiology of the hippocampus that nonetheless allow for the successful completion of hippocampus-

dependent tasks. This may indicate a convergence of mechanisms whereby both unstressed males and stressed males given a rest period are able to achieve the same behavioral outcome via different strategies. Although I used a more targeted approach to assess gene expression changes following the cessation of chronic stress, data from Chapters 4 and 5 suggest a similar phenomenon may be occurring in medial prefrontal cortex. That is, chronically stressed males given a rest period no longer have a deficit in extradimensional set-shifting compared to unstressed males; however, stressed males given a rest period have markedly higher expression of several genes related to glutamatergic and GABAergic function.

Given that comparatively little is known about the immediate effects of chronic stress on mPFC in females, it is unsurprising that almost nothing is known regarding changes that may arise during the post-stress rest period. Studies that have examined post-stress changes in females have yielded mixed findings. McFadden and colleagues (2011) found that chronic stress-induced behavioral facilitation on the Morris water maze in females persisted through a post-stress rest period. In contrast, Ortiz and colleagues (2015) found no behavioral change in performance on the radial arm water maze in females following chronic stress. However, following a rest period, chronically stressed females performed worse on the same task than unstressed females. I have shown that dendritic remodeling in prelimbic cortex is minimal both immediately following chronic stress and through a post-stress rest period (Chapter 2; Moench and Wellman, 2017). Further, attentional set-shifting performance was unaltered immediately following chronic stress and following a post-chronic stress rest period (Chapter 4). However, like in males, data from Chapter 5 indicate there are gene expression changes that arise following chronic stress. Chronic stress did not affect the expression of the AMPA receptor subunit GluR1 in females immediately after stress, but an increase in GluR1 expression emerged following a rest period. On the other hand, chronic stress resulted in an increase in the expression of parvalbumin, and this increase persisted through the post-stress rest period. Unlike in males, chronic stress did not alter the expression of NR1 or Gad67 in females, either immediately or after the rest period.

Thus, while changes in gene expression are present both immediately following chronic stress and following a post-chronic stress rest period in males and females, the pattern of changes is different between males and females. These data, combined with different changes in dendritic architecture and attentional set-shifting performance in males and females, indicate that chronic stress alters the structure and function of prelimbic cortex in a sex-specific manner. Further, the sex-specific changes that persist and/or emerge following the cessation of chronic stress likely contribute to sex differences in the response to a novel stress challenge.

Chronic stress results in sex-specific responses to a novel stress challenge.

Few studies have examined the effects of prior chronic stress in adulthood on responsivity to a novel stressor following a no-stress rest period. Early studies examining a novel stress challenge on the day following chronic stress showed stress-sensitization in males, whereby rats that underwent chronic stress had greater increases in plasma ACTH and corticosterone in response to an acute stress challenge compared to previously stress-naïve rats (Bhatnagar and Dallman, 1998). In contrast, a more recent study suggests the opposite: chronically stressed male rats had blunted levels of plasma ACTH and corticosterone following a novel acute stressor (Ostrander et al., 2006). This study also showed that this reduction in plasma corticosterone was also present when the acute stress challenge occurred following a 7 day rest period. In a follow up study, alterations in c-*fos* mRNA were found across a number of corticolimbic brain regions. Notably, some of these alterations were still present up to 30 days after the cessation of chronic stress (Ostrander et al., 2009). Further, Gray and colleagues (2014) investigated the effects of prior chronic stress on gene expression in the hippocampus following a novel stress challenge. They found that chronically stressed animals exposed to a novel stress challenge following a 21 day rest period had distinct differences in gene expression changes relative to either chronically stressed rats exposed to the acute stressor without a rest period, and previously stress-naïve

animals exposed only to the acute stressor. Together, these studies suggest that chronic stress can have both immediate and lasting effects on responsivity to novel stressors.

Data presented here expand on these findings in males, and begin to investigate if there are sex differences in how prior chronic stress modulates responsivity to a novel stress challenge, with a specific focus on the function of prelimbic cortex. In Chapter 3 (Moench et al., 2019), I showed that chronically stressed male rats have blunted c-Fos expression in prelimbic cortex following a novel stress challenge. This was the case both when the acute stressor occurred on the day after the cessation of chronic stress, and when it occurred following a 7 day rest period. In contrast, c-Fos expression in prelimbic cortex of chronically stressed female rats at both post-stress timepoints was no different than that of females exposed only to acute stress. In Chapter 5, I went on to show that there are also sex-specific changes in the expression of NR1, GluR1, Gad67, and parvalbumin in response to a novel stress challenge in chronically stress rats. Notably, changes in the expression of these genes were different depending on the presence of a rest period. In chronically stressed males not given a rest period, the expression of NR1, GluR1, and parvalbumin in response to acute stress was lower than that in males exposed only to acute stress. When given a rest period, the expression of these genes was comparable between chronically stressed males and males exposed only to acute stress. In females, the opposite pattern of change was found for GluR1 expression. Acute stress exposure on the day after chronic stress resulted in an increase in GluR1 expression, but this increase was not present when acute stress occurred following a rest period. While the relationships among changes in the expression of c-Fos and the glutamatergic and GABAergic genes examined in this dissertation are presently unclear, these findings do support the notion that chronic stress can lead to lasting changes in the response of prelimbic cortex to a novel stress challenge. Further, it is clear that there are striking sex differences in the pattern of changes that emerge in response to two-hit stress.

Perhaps the most surprising finding to emerge from the data presented in this dissertation is the sex-specific effects of two-hit stress on extradimensional set-shifting. In Chapter 4, I showed

that set-shifting performance is not altered in males following two-hit stress. In contrast, chronically stressed females have a profound deficit in extradimensional set-shifting following an acute stress challenge – but only when acute stress occurred following a rest period. Data from Chapter 3 provide an interesting hypothesis as to why this pattern emerged. In the paraventricular nucleus of chronically stressed females, c-Fos expression was markedly greater following an acute stress challenge. Notably, like the deficit in extradimensional set-shifting, this increase only occurred when acute stress followed a post-chronic stress rest period. In contrast, c-Fos expression in chronically stressed males at this same time was no different than that in males exposed only to acute stress. Thus, it is possible that enhanced c-Fos in the paraventricular nucleus of females may represent a facilitated response of the HPA axis to this novel stress challenge, in turn disrupting behavioral performance on the set-shifting task. Interestingly, Ortiz and colleagues (2015) showed that while chronic stress did not disrupt performance on a water maze version of the radial arm maze in females, more errors were made by chronically stressed females following a rest period. Although the authors offered no explanation of this finding, it is interesting to draw parallels between that finding and data presented here. Given that placing rats in water is often used as an acute stress manipulation in the form of the forced swim test (Gray et al., 2014), it is possible that having rats perform a water version of the radial arm maze introduced the addition of a mild stressor. Although highly speculative, the findings from Ortiz and colleagues (2015) interpreted in this light align well with the data presented here showing that behavior is disrupted in females, but not males, following two-hit stress.

Conclusions

In this dissertation I showed that not only does stress have immediate sex-specific effects on rat medial prefrontal cortex, but also that chronic stress can lead to sex differences in the function of medial prefrontal cortex in response to a novel stress challenge. These findings contribute to a growing interest in the lasting effects of stress on brain regions critical for executive

functioning and emotion regulation (Ortiz and Conrad, 2018), and support the notion that the process of “recovery” from chronic stress does not simply involve a return to an unstressed state. Instead, it is likely that exposure to chronic stress results in the recruitment of stress adaptation mechanisms that influence how an organism responds to future stressors. Further, this dissertation provides the first piece of evidence that this recruitment of stress adaptation mechanisms may be sex-specific. Thus, data from this dissertation do not support the notion that female rats are resilient to the effects of chronic stress or fail to adapt to chronic stress, but instead have different stress adaption mechanisms following chronic stress compared to males. This may represent a point of divergence in risk and resilience between males and females, whereby males may be buffered to some degree against the deleterious effects of multiple stressors. In contrast, females may be more susceptible to the compounding effects of stress. This, in combination with other known risk factors may increase susceptibility for the development of stress-related disorders. Understanding sex differences in the lasting effects of chronic stress, and how other known risk factors interact with post-stress changes will contribute to a better understanding of the etiologies of stress-related psychological disorders, especially those in which prevalence differs between men and women.

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Education

2019 **Indiana University, Bloomington, IN**
Ph.D. Neural Science, Psychology

2014 **Carthage College, Kenosha, WI**
B.A. Neuroscience, Psychology, summa cum laude

Research Training

Graduate Research Assistant (2014 - present)
Department of Psychological and Brain Sciences, Program in Neuroscience
Indiana University – Bloomington, IN
Principal Investigator: Cara L. Wellman, Ph.D.
Dissertation: Sex differences in the effects of two-hit stress on the structure and function of rat medial prefrontal cortex.

Immunohistochemistry | Golgi Histology | Dendritic Morphology Analyses | ELISA | Stereology | Densitometry | Stereotaxic Surgery | qPCR

Undergraduate Research Assistant (2012 - 2014)
Program in Neuroscience — Carthage College — Kenosha, WI
Principal Investigator: Daniel P. Miller, Ph.D.
Focus: Effect of amygdala lesions on strain differences in avoidance learning.

Stereotaxic Surgery | Rodent Behavioral Testing | Tissue Collection | Gross Histology Analyses

Publications and Articles in Press

Moench, K.M., Breach, M.R., Wellman, C.L. (2019). Chronic stress produces enduring sex- and region-specific alterations in novel stress-induced c-Fos expression. *Neurobiology of Stress,* 10, 100147*.*

Godar, S.C., Mosher, L.J., Melis, M., Scheggi. S., **Moench, K.M.**, et al. (2019). Gene-environment interactions in antisocial behavior are mediated by early-life 5-HT2A receptor activation. *Neuropharmacology*. *In Press.*

Wellman, C.L., **Moench, K.M.** (2019). Preclinical studies of stress, extinction, and prefrontal cortex: Intriguing leads and pressing questions. *Psychopharmacology,* 236, 59-72*.*

Wellman, C.L., Bangasser, D. A., Bollinger, J. L., Courtellier, L., Logrip, M., **Moench, K.M.**, Urban, K. (2018). Sex differences in risk and resilience: Stress effects on the neural substrates of emotion and motivation. *The Journal of Neuroscience*, 38, 9423-9432.

Moench, K.M., Wellman, C.L. (2017). Dendritic reorganization in medial prefrontal cortex of male and female rats following recovery from chronic stress. *Neuroscience*, 357, 145-159.

Moench, K.M., Maroun, M., Kavushansky, A., Wellman, C.L. (2016). Alterations in neuronal morphology in infralimbic cortex predict resistance to fear extinction deficits following acute stress. *Neurobiology of Stress,* 3, 23-33.

Moench, K.M., Wellman, C.L. (2015). Stress-induced alterations in prefrontal dendritic spines: Implications for post-traumatic stress disorder. *Neuroscience Letters,* 601*,* 41-45.

Manuscripts Under Review and In Preparation

Miller, J. R., Cook-Snyder, D.R., Buchholz, K., Evenhouse, A., Nicosia, T., Grove, A., Regetz-Mularney, T., Patschorke, E., **Moench, K.M.**, Miller, D.P., Martino, P.F. Developing a low budget system to mix, store, and deliver enhanced respiratory gasses for human research in liberal arts college setting. *Under Review.*

Breach, M.R., **Moench, K.M.**, Wellman, C.L. Chronic stress induces sex-specific dendritic reorganization in medial prefrontal cortex of adult rats that experienced social instability in adolescence. *In preparation.*

Poster Presentations

Moench, K.M., Wellman, C.L. (2018). Sex-specific effects of two-hit stress on behavioral flexibility in rodents. *International Behavioral Neuroscience Society Annual Meeting.*

Breach, M.R., **Moench, K.M.**, Wellman, C.L. (2018). Sex-specific dendritic reorganization after chronic stress in adult rats that experienced social instability as adolescents. *25th Annual CISAB IU Animal Behavior Conference.*

Moench, K.M., Wellman, C.L. (2017). Prior chronic stress exposure alters medial prefrontal cortex response to a novel stressor in a sex-dependent manner. *Society for Neuroscience abstracts.*

Moench, K.M., Wellman, C.L. (2017). Chronic stress-induced changes in neural responses to a subsequent stressor are sex-dependent. *CISAB ambassador to the Center for Behavioral Neuroscience Annual Brain and Behavior Spring Retreat at Georgia State University.*

Moench, K.M., Wellman, C.L. (2016). Lasting sex differences in chronic stress-induced patterns of fos and brain-derived neurotrophic factor expression in prelimbic cortex in response to a novel acute stressor. *Society for Neuroscience abstracts.*

Moench, K.M., Wellman, C.L. (2015). Dendritic reorganization in medial prefrontal cortex of male and female rats following recovery from chronic stress. *Society for Neuroscience abstracts.*

Moench, K.M., Miller, D.P, Allen, M.T., Pang, K.C.H., Servatius, R.J. (2014). Amygdala lesions impair lever press avoidance acquisition of inbred Wistar-Kyoto rats, but not outbred Sprague Dawley rats. *Society for Neuroscience abstracts.*

Moench, K.M., Resch, Z.J., Wilson, J.J., Miller, D.P., Pang, K.C.H, Servatius, R.J. (2013). Amygdala lesions impair signaled lever press avoidance acquisition in Wistar Kyoto and Sprague Dawley rats. *Society for Neuroscience abstracts.*

Moench, K.M., Wilson, J.J, Miller, D.P., Pang, K.C.H, Servatius, R.J. (2013). Amygdala lesions impair signaled lever press avoidance acquisition in Wistar Kyoto and Sprague Dawley rats. *Pavlovian Society Annual Meeting abstracts.*

Oral Presentations

Moench, K.M., Wellman, C.L. (2018). When it's over, is it really over? Sex differences in the lasting effects of chronic stress on rat medial prefrontal cortex. *Society for Neuroscience Annual Meeting.*

Moench, K.M., Wellman, C.L. (2018). Sex differences in risk and resilience: Recovery of stress-induced dysfunction of prefrontal cortex in male and female rats. *International Behavioral Neuroscience Society Annual Meeting.*

Moench, K.M., Wellman, C.L. (2016). Sex-specific changes in medial prefrontal cortex following recovery from chronic stress. *Clinical Science Colloquium Series, Indiana University.*

Moench, K.M., Maroun, M., Wellman, C.L. (2014). The effects of acute stress on spine density in rat medial prefrontal cortex. *National Conference on Undergraduate Research, University of Kentucky.*

Moench, K.M., Maroun, M., Wellman, C.L. (2013). The effects of acute stress on spine density in rat medial prefrontal cortex. *Midstates Consortium for Undergraduate Research, Washington University.*

Grants, Fellowships, and Funding

NICHD T32 Common Themes in Reproductive Diversity Training Fellowship, 2018 – 2019
Indiana University, Bloomington

NIMH T32 Clinical Translational Science Training Fellowship, 2016 – 2018
Indiana University, Bloomington

Harlan Scholars Summer Research Fellowship, 2017
Indiana University, Bloomington

President’s Graduate Diversity Fellowship, 2015 – 2016
Indiana University, Bloomington

Program in Neuroscience Fellowship, 2014 – 2015
Indiana University, Bloomington

Neuroscience Department Fellowship, 2012 – 2014
Carthage College, Kenosha, WI

Faculty Honor’s Scholarship, 2012
Carthage College, Kenosha, WI

Honors and Awards

J.R. Kantor Graduate Award for Outstanding Advanced Graduate Students, 2019
Dept. of Psychological and Brain Sciences, Indiana University, Bloomington

International Behavioral Neuroscience Society Travel Award, 2018
Annual Meeting, Boca Rotan, FL

Women in Science Travel Award, 2017
Indiana University, Bloomington

Qualifying Exam Commendation, 2016
Dept. of Psychological and Brain Sciences, Indiana University, Bloomington

College of Arts and Sciences Travel Award, 2015, 2016, 2017, 2018
Indiana University, Bloomington

NSF Graduate Research Fellowship – Honorable Mention, 2014
Indiana University, Bloomington

NSF Research Experience for Undergraduates, 2013
Indiana University, Bloomington

Teaching, Mentorship, and Outreach Opportunities

Associate Instructor
Professional Ethics for the Bio-behavioral Sciences (Fall 2018)
Methods in Experimental Psychology, Laboratory (Fall 2016)

Undergraduate and Technician Training/Mentorship
Michaela Breach (undergraduate, 2017-present)
Violetta Szalavari (technician, 2015-2018)
Arianna Gutierrez (technician, 2015-2017)
Shakeera Walker (undergraduate, summer 2015)

Training in immunohistochemistry, basic microscopy, dendritic morphology analyses, and rodent behavioral testing.

Outreach
Science Fest, volunteer, 2015, 2018
Indiana University, Bloomington

Professional Service

Ad hoc reviewer – *Behavioral Brain Research*

Committee Chair, Animal Behavioral Conference, 2015 – 2018
Undergraduate Poster Competition

Affiliations and Professional Training

Affiliations

International Behavioral Neuroscience Society
Student Member, 2018-present

Society for Neuroscience
Student Member, 2013-present

Center for the Integrative Study of Animal Behavior (CISAB)
Indiana University, Bloomington
Member, 2013-present

Professional Training

Indiana University Kelley School of Business
Certificate in the Business of Life Sciences, 2018

Research Ethics Training Workshop
Poynter Center, Indiana University, 2015

Practical Training Course in Confocal Microscopy and Quantitative Histology
NeuroRenew, 2015